REMARKS

Claims 13-16 and 26-31 were pending in the application. Accordingly, upon entry of the amendments presented herein, 13-16 and 26-31 will remain pending in the application.

Claim 13 has been amended to specify a method for diagnosing an autoimmune disease, by taking a sample from a patient, testing the sample for IgA antibodies against human tissue transglutaminase, testing the sample for IgA antibodies against at least one other transglutaminase molecule selected from the group consisting of a-subunit of factor XIII (FXIIIA), keratinocyte transglutaminase (TGk), transglutaminase X (TGx), epidermal transglutaminase (TGe) and Band 4.2; and correlating significantly increased amounts of the IgA antibodies specific for human tissue transglutaminase and/or IgA antibodies specific for at least one other transglutaminase molecule selected from the group consisting of a-subunit of factor XIII (FXIIIA), keratinocyte transglutaminase (TGk), transglutaminase X (TGx), epidermal transglutaminase (TGe) and Band 4.2, as compared to a control sample, with a diagnosis of a gluten sensitive enteropathic autoimmune disease. Support for this amendment can be found throughout the specification and claims as originally filed. Specifically, support is available at page 3 (lines 8-13); page 10, line 28 through page 17, line 22; page 22 (lines 4-6); Original claims 5-7.

Claims 15-16 and 26-31 have been withdrawn as encompassing non-elected species. However, as acknowledged by the Examiner, Applicants will be entitled to consideration of additional non-elected species encompassed by the generic claim. It is further Applicants' understanding that the species election was for search purposes only and that the search will be extended to additional species upon a finding of allowability of the elected species.

No new matter has been added. The foregoing claim amendments should in no way be construed as an acquiescence to any of the Examiner's rejections and were made solely in the interest of expediting prosecution of the application. Applicants reserve the right to pursue the claims as originally filed in this or a separate application(s).

Acknowledgment of the Examiner's Withdrawal of Certain Rejections

Applicants acknowledge with appreciation the withdrawal of the (a) the prior rejection of claim 32 as lacking written description under 35 U.S.C. § 112, first paragraph; (b) the prior rejection of claims 13, 14 and 32 under 35 U.S.C. § 112, first paragraph, as lacking enablement; and (c) the prior rejection of claim 32 under 35 U.S.C. § 112, second paragraph as allegedly being indefinite.

Rejection of Claims 13 and 14 Under 35 U.S.C. § 102(b) or (e)

The Examiner has maintained the rejection of claims 13 and 14 under 35 U.S.C. § 102(b) or (e) as being anticipated by Schuppan *et al.* (WO 98/03873 or US 6,319,726). The Examiner relies on Schuppan *et al.* for teaching "methods of detecting antibodies from body fluids by means of an immune reaction with tissue transglutaminase," such as human tissue transglutaminase. The Examiner also relies on Schuppan *et al.* for teaching "that the tissue transglutaminase can be immobilized and used to detect antibodies in a sample for diagnosing celiac disease (sprue) (gluten sensitive enteropathic autoimmune disease)" and that such a method "is used to detect IgA antibodies." The Examiner further asserts that the antibodies to be detected by Schuppan *et al.* "are the same as the antibodies detected by [A]pplicant[s]" and that the "antibodies of Schuppan *et al.* would be cross reactive with other antigens and would inherently be against TGe."

Applicants respectfully traverse this rejection. Notwithstanding, to expedite prosecution and allowance of the pending claims, Applicants have amended claim 13 to clarify that the presently claimed method includes (1) testing a sample for the presence of IgA antibodies against human tissue transglutaminase, (2) testing the sample for IgA antibodies against at least one other transglutaminase molecule (*i.e.*, TGe, TGk, TGx, FXIIIA (a-subunit) and/or Band 4.2), and (3) correlating significantly increased amounts of the IgA antibodies specific for human tissue transglutaminase and/or IgA antibodies specific for at least one other transglutaminase molecule selected from the group consisting of a-subunit of factor XIII (FXIIIA), keratinocyte transglutaminase (TGk), transglutaminase X (TGx), epidermal transglutaminase (TGe) and Band 4.2, as compared to a control sample, with a diagnosis of a gluten sensitive enteropathic autoimmune disease.

In contrast to the presently claimed method, which involves detection of IgA antibodies against human tissue transglutaminase and at least one other transglutaminase molecule, Schuppan *et al.* teach methods of identifying coeliac disease/non-topical sprue disease in a subject by detecting the binding of antibodies in body fluids to a single target antigen (*i.e.*, tissue transglutaminase (tTG)). Schuppan *et al.* teach that tTG is the autoantigen of sprue and describe methods of diagnosing sprue or celiac disease using antibodies that bind to tTG. Despite the widespread occurrence of transglutaminase enzymes in the body, Schuppan *et al.* specifically limited their method of diagnosis to the detection of IgA antibodies against tTG, since the authors of Schuppan *et al.* believed that tTG was the only transglutaminase enzyme which could come into direct contact with the gluten contained in food.

However, as taught in Applicants' specification at page 3, line 28 through page 4, line 7, Schuppan's discovery of tTG (also known as TGc) as the autoantigen of sprue did not answer the question why only a proportion of patients with coeliac disease also shown symptoms of dermatitis herpetiformis (DH). As part of the present invention, Applicants discovered that other transglutaminases also play a role in gluten sensitive enteropathic autoimmune diseases. Accordingly, the presently claimed method of diagnosis involve testing a sample for the presence of IgA antibodies against human tissue transglutaminase and then further testing the sample for IgA antibodies against at least one other transglutaminase molecule selected from the group consisting of TGe, TGk, TGx, FXIIIA (a-subunit) or Band 4.2. Schuppan *et al.* do not teach or suggest further testing a sample for the presence of different IgA antibodies specific for TGe, TGk, TGx, FXIIIA (a-subunit) or Band 4.2, as presently claimed.

In addition, contrary to the Examiner's assertion, the antibodies of Schuppan *et al.* would not inherently be cross-reactive with TGe, TGk, TGx, FXIIIA (a-subunit) or Band 4.2. Specifically, as evidenced by the enclosed publication by Aeschlimann *et al.*, (*Thromb Haemost.*, 71(4):402-15. (1994); enclosed herewith as Appendix A), the various transglutaminases are immunologically distinct molecules, which differ from tTG and one another based on their physical properties, their location in the body and their primary structures. In fact, FXIIIA (a-subunit) is only 75% homologous to tTG and TGe, TGk, TGx, and Band 4.2 are even less homologous.

Moreover, as evidenced by the enclosed publications by Marietta *et al.* (*J. Invest. Dermatol.*, 128(2):332-5 (2008); Appendix B), Hull *et al.* (*Br. J. Dermatol.*, 159(1):120-4 (2008); Appendix C) and Sardy *et al.* (*J Exp Med.*, 195(6):747-57 (2002); Appendix D), the antibodies which bind tTG and TGe are, indeed, different antibodies and support the diagnostic relevance of separately testing for the presence of tTG and TGe in patients suspected of suffering from a gluten sensitive enteropathic autoimmune disease. Specifically, as taught by Marietta *et al.*, patients afflicted with dermatitis herpetiformis were found to have "TGe-specific IgA that fell into two groups"... "[o]ne antibody group bound to TGe exclusively, whereas the second antibody group was cross-reactive and bound to both tTG and TGe" (see page 332). Additionally, Hull *et al.* confirm that patients with dermatitis herpetiformis have elevated levels of antibodies against tTG, as well as antibodies against TGe. Further as described by Sardy *et al.*, TGe, not tTG, is the autoantigen of dermatitis herpetiformis. As such, the Examiner is incorrect in asserting that the antibodies taught by Schuppan *et al.* are inherently cross-reactive with tTG and TGe, TGk, TGx, and Band 4.2.

Accordingly, since Schuppan *et al.* fail to teach or suggest each and every element of the present invention (*i.e.*, testing a sample for the presence of IgA antibodies against human tissue transglutaminase, testing the sample for IgA antibodies against at least one other transglutaminase molecule (*i.e.*, TGe, TGk, TGx, FXIIIA (a-subunit) and/or Band 4.2) and correlating significantly increased amounts of the IgA antibodies specific for human tissue transglutaminase and/or IgA antibodies specific for at least one other transglutaminase molecule selected from the group consisting of a-subunit of factor XIII (FXIIIA), keratinocyte transglutaminase (TGk), transglutaminase X (TGx), epidermal transglutaminase (TGe) and Band 4.2, as compared to a control sample, with a diagnosis of a gluten sensitive enteropathic autoimmune disease) claims 13 and 14 are novel over the cited reference. As such, Applicants respectfully request the Examiner to reconsider and withdraw this rejection.

CONCLUSION

In view of the above amendments and remarks set forth above, it is respectfully submitted that this application is in condition for allowance. If there are any remaining issues or the Examiner believes that a telephone conversation with Applicants' Attorney could be helpful in expediting prosecution of this application, the Examiner is invited to call the undersigned at (617) 227-7400.

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Respectfully submitted,

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Review Article

Transglutaminases: Protein Cross-Linking Enzymes in Tissues and Body Fluids

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Introduction

Transglutaminases catalyze the posttranslational modification of proteins referred to as the R-glutaminyl-peptide, amine-γ-glutamyl transferase reaction (EC 2.3.2.13), which leads to the formation of an isopeptide bond either within or between polypeptide chains. It appears that nature has not provided an enzyme for the cleavage of the transglutaminase cross-link in proteins. The action of these enzymes results therefore in the formation of "irreversibly" cross-linked, often insoluble supramolecular structures. In vertebrates, transglutaminases form a large protein family and have a wide distribution amongst tissues and body fluids. Proteins modified by transglutaminases are found throughout the organism, *e. g.* in fibrin clots formed in hemostasis and wound healing, in cell membranes of terminally differentiated erythrocytes, in extracellular matrices, and in the cornified envelope of the epidermis. Transglutaminases occur early in evolution and enzymes with a similar function to vertebrate transglutaminases have been found in invertebrates, in plants, in unicellular eucaryotes and in bacteria. While transglutaminases in higher animals always require Ca^{2+} for activity, this requirement is less stringent in plants and microorganisms. Recently, the primary structure of a bacterial transglutaminase was determined (1) and it was revealed that these enzymes evolved as a separate lineage from the eucaryotic transglutaminases. For historical reasons several different names are used to describe a single transglutaminase gene product. The characterized gene products and the different designations are summarized in Table 1. The physiological function of the various transglutaminases is not well understood with the exception of the comparatively well characterized factor XIIIa. The widespread occurrence and high degree of conservation among transglutaminases could indicate that covalent protein cross-linking plays a more important role in nature than presently appreciated.

Enzymology

The transglutaminase-catalyzed Ca^{2+}-dependent acyl-transfer reaction results in the formation of new γ-amide bonds between γ-carboxamide groups of peptide-bound glutamine residues and various primary amines (Fig. 1 [for review see 2, 3]). A glutamine residue serves as acyl-donor and the most common acyl-acceptors are ε-amino groups of peptide-bound lysine residues or primary amino groups of some naturally occurring polyamines, like putrescine or spermidine. In the first case the reaction results in the formation of γ-glutamyl-ε-lysine cross-links either in or between proteins, whereas the reaction with polyamines results in protein modifications possibly affecting the biological activity or turnover of the target protein, but not in polymer formation. The reaction is a multistep process, in which the active site cysteine reacts first with the γ-carboxamide-group of a glutamine residue to form the acyl-enzyme intermediate under release of ammonia. In a second step, the complex reacts with a primary amine to form an isopeptide bond and liberate the reactivated enzyme. The driving force for the reaction is supplied by the release of ammonia and its subsequent protonation, occurring readily under physiological conditions. The amine incorporation proceeds with a Michaelis-Menten type of saturation kinetics and should, in a strict sense, be considered reversible as an exchange of amines linked to protein-bound glutamine residues may occur even though this reaction is likely to be negligible under physiological conditions.

Transglutaminases also exhibit catalytic activity towards certain esters such as p-nitrophenyl-acetate and catalyze their hydrolysis by reaction with H_2O, at least under *in vitro* conditions (2, 4). However, reaction of H_2O or alcohols with the acyl-enzyme intermediate clearly occurs at much slower rate than with primary amines.

The number of proteins acting as glutaminyl substrates is restricted, as both primary structure (including charge) and conformation determine whether a glutamine residue is reactive or not (2, 5, 6, 7). In contrast, the tolerance to structural differences in acyl-acceptors is considerable with preference for branched chain aliphatic amines with a methylene chain equal in length to the side chain of a lysine residue (2, 3). Amino acid residues adjacent to the lysine do not have a pronounced influence on the substrate properties, which explains why protein-bound lysine residues and small primary amines serve equally well as amine donors. Thus, in a particular tissue only a small subset of proteins are glutaminyl substrates for the enzyme, whereas most proteins are able to contribute ε-amino groups of lysine residues to serve as acyl acceptors in the cross-linking reaction. Furthermore, the specificity for different glutaminyl substrates differs between transglutaminases, as shown by the structural difference in fibrin polymers formed by the action of TG_C and factor XIIIa (8) and by the different affinities for amine incorporation by these enzymes into synthetic peptide variants of the amine acceptor site in β-casein (5, 6). Distinct transglutaminases may recognize the same protein as substrate, but often with different affinity and/or with specificity for different glutamine residues. The structural requirements of glutaminyl substrates are more stringent for factor XIIIa than for TG_C (5, 6, 9).

Structure of Transglutaminases

1. Factor XIII. The subunit structure of plasma factor XIII has been established by electron microscopy of rotary shadowed samples combined with sedimentation equilibrium centrifugation. In plasma, it is a tetramer composed of two filamentous b-subunits and a dimer of non-covalently associated a-subunits and when occurring intracellular-

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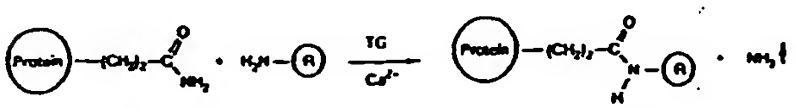

Fig. 1 Reaction Scheme of the Transglutaminase-catalyzed Transfer Reaction between the γ-Carboxamide Group of the Peptide-bound Glutamine Residue and a Primary Amine. *R* represents either a protein-bound lysine residue in the reaction resulting in the formation of a protein cross-link or an aliphatic side chain of variable structure in the reaction resulting in the incorporation of a polyamine into the protein

ly, a dimer of only the a-subunit (10). Factor XIII is a zymogen that is activated to factor XIIIa by thrombin cleavage of the a-subunit (see below).

The primary structure of human placenta factor XIII a-subunit has been established by cDNA-cloning (11, 12) and protein sequencing (13). The mature protein is produced by cleavage of the NH_2-terminal methionine and acylation of the following serine residue and consists of 731 amino acids with a molecular mass of ~83 kDa (13, 14). The active site cysteine is located at position 314 (Fig. 2, *cross* [2]). The cDNA sequence indicates that the a-subunit of factor XIII does not contain a typical hydrophobic leader sequence for secretion (11, 12). The absence of glycosylation and disulfide bonding (13), despite numerous potential sites for N-linked glycosylation as well as free cysteines, is consistent with it being a typical cytoplasmic protein. In fact, factor XIII is found in monocytes, macrophages, megakaryocytes, platelets, uterus and placenta in the intracellular dimeric form (a_2) (for review see 15). The tetrameric plasma enzyme (a_2b_2) results from secretion of the a-subunit by an unknown mechanism. Platelets, peripheral blood monocytes and the liver have been suggested to be the primary sites of plasma factor XIII synthesis (16, 17). Expression of factor XIII a-subunit as a fusion protein with the preprodomain of a secretory protein in yeast yielded glycosylated variants of factor XIII that were largely retained and degraded in the endoplasmic reticulum (18). Apparently, targeting of factor XIII a-subunit to the conventional secretory pathway leads to aberrant posttranslational modifications.

The primary structure of the b-subunit of human factor XIII has been determined by cDNA cloning (19). It is composed of 641 amino acids and contains about 8.5% carbohydrate. The resulting molecular mass is approximately 80 kDa. The gene encodes, in addition, a 20 amino acid leader sequence common to conventional secretory proteins (Fig. 3 [20]).

Sequence analysis revealed that the b-subunit of factor XIII is entirely composed of 10 tandem repeats of about 60 amino acids each, which are called GP-I structures as they were first discovered in β_2-glycoprotein I (21) or sushi-structures because of their shape (Fig. 3 [20]). The repeats contain four conserved cysteine residues that form characteristic disulfide bonds between the 1st and 3rd, and the 2nd and 4th cysteine (21). Proteins containing a variable number of this type of repeat are found in vertebrates as well as in invertebrates and participate in diverse biological systems, *e. g.* in the complement cascade, in blood coagulation and as cell surface receptors in lymphocyte regulation (for review see 20). As their physiological function most often involves binding to other proteins it appears likely that the GP-I repeat serves as a protein-binding module. The recently solved gene structure of the factor XIII b-subunit (22) revealed that each repeat is encoded by a single exon (Fig. 3), consistent with the view that exon shuffling and duplication during evolution led to the current distinct gene products (20).

Table 1 Transglutaminase terminology. The designations used throughout the text are underlined

Gene Product	Alternative Designations
Plasma transglutaminase	Factor XIII(a), fibrin stabilizing factor, Laki-Lorand factor, fibrinoligase
Tissue transglutaminase	TG_C, cytosolic-, endothelial-, erythrocyte-, liver transglutaminase, transglutaminase type II
Keratinocyte transglutaminase	TG_K, particulate transglutaminase (transglutaminase B[1]), transglutaminase type I
Epidermal transglutaminase	TG_E, bovine snout-, callus-, hair follicle transglutaminase, transglutaminase type III
Prostate transglutaminase	TG_P, dorsal prostate protein 1, major androgen-regulated prostate secretory protein, vesiculase
Erythrocyte band 4.2	B4.2, erythrocyte membrane protein band 4.2
Hemocyte transglutaminase	TG_H, *Limulus* transglutaminase, invertebrate factor XIII analogue?
Annulin	Invertebrate keratinocyte transglutaminase analogue?

[1]) A particulate fraction associated transglutaminase, termed transglutaminase B, present in lung, liver, and chondrosarcoma has been shown to be identical to TG_K (79). An association of TG_C with particulate fractions has been observed and is due to an interaction with membrane bound proteins (109)

2. *Tissue transglutaminase.* Molecular cloning of TG_C of human (23), bovine (24), mouse (23), guinea pig (25) and chicken (26) origin, revealed a polypeptide of 685–691 amino acids with a molecular mass of ~77 kDa (Fig. 2). The conservation between species is moderate (88% from bovine, 84% from mouse, 80% from guinea pig and 65% from chicken to human). The active site has been shown to involve cysteine 276 in the guinea pig enzyme (Fig. 2, *cross* [25, 27]). TG_C is not glycosylated (2) nor disulfide bonded (27, 28), although it contains 17 cysteine residues and 5–6 potential sites for N-linked glycosylation. Consistent with these findings, TG_C lacks a hydrophobic leader sequence found in secreted proteins and the NH_2-terminus of TG_C is blocked by removal of the initiator methionine and subsequent acetylation of the penultimate alanine residue (29). The N^{α}-acetyl group does not influence the catalytic activity of the enzyme (30) and might therefore have a different function. It has recently been suggested that N^{α}-acylation is a signal for "alternative" secretion of proteins (31). There is evidence for externalization of TG_C in a variety of tissues

Fig. 2 Comparison of the Amino Acid Sequences of Human Factor XIII a-Subunit, Human TG_C, Human TG_K, Human TG_E, Rat TG_P, Human B4.2, *Limulus* TG_H and Grasshopper Annulin. The sequences of TG_C, TG_K and TG_E show the positions of amino acid variation between different species as small letters (TG_C comprises sequences of human, bovine, mouse, guinea pig and chicken with identical amino acids in three species taken as conserved; TG_K such of human and rat; and TG_E such of human and mouse). Dashes indicate gaps inserted for optimal sequence alignment, underlined residues represent amino acids conserved in ≥4 different transglutaminases, the cross indicates the active site Cys, arrows mark the protease cleavage sites in factor XIII a-subunit (Arg-37) and TG_E (Ser-469), and triangles mark exon-intron boundaries in the genes. Asterisks and open circles at the bottom of the aligned sequences indicate positions that are occupied by identical or chemically similar amino acids in all transglutaminases. Alternative splicing of exon I of the B4.2 gene results in insertion of the sequence GEPSQRSTGLAGLYAAPAASPVFIKGSGMD after Gln-3 in the protein (B4.2L)

```
TGK                                                                    MdCPRSDVCRWCgnPI   16

FXIII                                    SETSRTAFCGRRAVPPNNSNAAEDDLPTVELQGVVPRGV   39
TGK    QpPTTPSPEPEPd----gRSRRGGCRSFWARCCGCCSCrNaADDDWGPEPSdSRgRGsSS---GtRrpgSRGsDS---RRPvSRGSGVNAAGDGT  101
TGH                       YCFGRGNHFRNRSTRYRRRPRYRAENYHSYHLDLLENHEEFGRNWWCTPESHQPD   56
Annu                      GNCCSTFRAVFKPNEGSGGGIPLMPVRGGSTRRPDSLPKPPAAVVPSPPSPGDVPDAC   58

FXIII  NLQEFLNVTSVHLFKERWDTNKVDHHTDKYEN------NKLIVRRGQSFYVQIDFS---RPYDPRRDLFRVEYVI--GRYPQENKGTYIPVPIVS  123
TGC       AEELVLERCDLELEt---NGRDHHTADLCR------EKLVVRRGQPFWLTLHFEG--RnYEASVDSLTFsvVT--GPaPSqEAGTKARFPLrD   80
TGK    IRECHLVVNGVDLLsSRSDQNRREHHTDEyEY------DELIvRRGQPFHmlLlLs---RtYESS-DRItLELLI--GNNPEVGKGTHVIIPVGK  184
TGE      aALgvQsiNWQkal---NRqAHHTDKFSS------QeIIRRGQnfqvLiimN---kgLgSn-ErLeFIdtT--GPyPSESAmTKAVFplSn   77
TGP    DSRNNLVVYSVNLEKKL---NAAAHHTIEYQT------QKLVLRRGQIFSLKVHLN---RPLQSH-DELKLIFNT--GHNHPF--YTVELDPMTS   78
B4.2     GQALGIKSCDFQAAR---NNEEHHTKALSS------RRLFVRRGQPFTIILYFRAPVRAFLPALKKVALTAQT--GEQPSKINRTQATFPISS   82
TGH    SGPSSLQVESVELYTRD---NAREHNTFMYDLVDGTK-PVLILRRGQPFSIAIREK---RNYNPQQDRLKLEIGF--GQQPLITKGTLIMLPVSG  142
Annu   VAPEVASVKEVDVLLAE---NGDAHRTRHYELMDREKEPRLVVRRGQPFAVSVTLS---RPYNPDIDAISFVFTVEDAEKPSYGQGTLVAVPLLA  147

FXIII  EL----QSGKWGAKIVMREDRSVRLSIQ-SSP-KCIVGKFRMYVAVWT----PYGVLRTSRNPETDTYILFNPWCEDDAVYLDNEKEREEYVLND  208
TGC    AV----EEGdWtAtvvDQQDCTLSLqLt-TPA-NAPIGLYRLSLEAST-------GYQGSSFVLGHFILLFNAWCPADAVYLDSEEERQEYVLTQ  162
TGK    G-----GSGGWKAQVvKasGqNLnLRVH-TSP-NAIIGKFQFTVRTqS---dAGEFQLPFDPRNEIYILFNPWCPEDIVYVDHEDWRQEYVLNE  268
TGE    g-----SsGGWsAvLqAsnGNcLtIsIs-SPa-SAPIGrYTmalqIfS-------qGgISSVkLGTFILLFNPWLnvDsVFMGNHAERcEYVqEQ  158
TGP    Y-----RSKGWQVKIAKQSGVEVVLNVI-SAA-NAVVGRYTMNVNEFD--------------AGVEFLLLNPWCSDDSVFMASEEDRAEYVLND  151
B4.2   LG----DRKWNSAVVEERDAQSWTISVT-TPA-DAVIGHYSLLLQ-------VSGRKQLLLGQFTLLFNPWNREDAVFLKNEAQRMEYLLNQ  161
TGH    SDTFTKDKTQWDVRLRQHDGAVITLEIQ-IPAAVA-VGVWKMK:VSQLTSEEQPNVSAVTHECKNKTYILFNPWCKQDSVYMEDEQWRKEYVLSD  235
Annu   KGAE---SGAANWAVLDSSADD--ILRIQITPAADAIVGKWKMD:DTKL-----KNDGAVSYSYKDPFYILYNPWCRQDQVFLEGEELLQEYVLND  233

FXIII  IGVIFYGEVNDIKTRSWSYGQFEDGILDTCLYVMDRA-------QMDLSGRGNPIKVSRVGSAMVNAKDDEGVLVGSWDNIYAYGVPPSAWTGSV  296
TGC    QGFIYQGSAKFIKnIPWNFGQFEqDGILDICLiLLDvNPKFLKNAGRDCSRRSSPVYVGRVgSgMVNCNDDQGVLLGRWDNNYgDGvSPMSWIGSV  257
TGK    SGRIYYGTEAQIGERTWNYGQFDHGVLDACLYILDRR-------GMPYGGRGDPVnVSRViSAMVNSLDDNGVLIGNWsGDYSRGTNPSAWVGSV  356
TGE    aGIIfvGSTNRIGMIGWNFGQFEEDILsIcLsILDRSLNFRRDaaTDVAsRNDPKYVgRVLSAMINsNDDNGVIaGNWSGcYTGGrDPRsWdGSV  253
TGP    TGYMYMGFAKQIKEKPWTFGRLRS-TLELLLPIVDPF-------GAQ--GNAEPVLVSRAICTMMCAANNFGVLVGNWTGDYSNGTAPYVWASSV  236
B4.2   NGLIYLGTADCIQAESWDFGQFEGDVIDLSLRLLSK-------DKQVEKWSQPVHVARVLCALLHFLKEQRVLPTPQTQATQEGALLNKRRGSV  248
TGH    VGKIFTGSFKQPVGRRWIFGQFTDSVLPACMLILERS-------GLDYTARSNPIKVVRAISAMVNNIDDEGVLEGRWQEPYCDGVAPWMWTGSS  323
Annu   TGLIWRGSYNRLRPCVWKYAQFEKEILDCALYLVSKIG------GVRPSECGDPVRVCRAISAAVNSPDDNGAVHGNWSNDYGGGTPPTKWIGSM  322

FXIII  DILLEYRSSEN-PVRYGQCWVFAGVFNTFLRCLGIPARIVTNYFSAHDNDANLQMDIFLEEDGNVNSKLTKDSVWNYHCWNQAWMTRPDLPVG--  388
TGC    DILRRWKnhGCQRVKYGQCWVFAAVACTVLRCLGIPTRVVTNYNSAHDQNSNLLIEYFRNEFGEIqgd-KSEMIWNFHCWVESWMTRPDLQPG--  349
TGK    EILLSYLRTGY-SVPYGQCWVFAGVTTTVLRCLGLATRTVTNFNSAHDTDTSLTMDIYFDENMKPLEHLNHDSVWNFHVWNDCWMKRPDLPSG--  448
TGE    EILKNWKKSGFsPVryGQCWVFAGTLNTaLRsLGipSRVITNFNSAHDTDRNLSVDVYYDPMGNPLdK-GSDSVWNFHVWNEGWFVRsDLGpP--  345
TGP    PILQQHYITRM-PVRFGQCWVFSGVLTTALRAVGIPARSVTNFESAEDTEKNLRVDIYLDESGKTIPHLTKDSVWNFHMWTDAWMKRQDLPQG--  328
B4.2   PILRQWLTGRGRPVYDGQAWVLAAVACTVLRCLGIPARVVTTFASAQGTGGRLLIDEYYNEEGLQNGEGQRGRIWIFQTSTECWMTRPALPQG--  341
TGH    AILEKYLKTRGVPVKYGQCWVFAGVANTVSRALGIPSRTVTNYDSAHDTDDTLTIDKWFDKNGDKIEDATSDSIWNFHVWNDCWMARPDLPTG--  416
Annu   KILQQFYKNKK-PVKYGQCWVFAGVLTTVCRALGLPARTVTTYSAAHDTQNSLTVDQYFVDDKGEIMEEMNSDSIWNFHVWTEVWMERPDLMPGDG  416

FXIII  --FGGWQAVD-STPQENSDGMYRCGPASVQAIKHGHVCFQFDAPFVFAEVNSDLIYITAKK----DGTHVVENVDATHIGKLIVTKQIGGD----  472
TGC    --YEGWQALD-PTPQEKSEGTYCCGPVPVRAIKEGDLSTKYDAPFVFAEVNADVVDWIqQd----DGSvhKSINrSLiVGLKISTKSVGRD----  433
TGK    --FDGWQVVD-ATPQETSSGIFCCGPCSVESIKNGLVYMKYDTPFIFAEVNSDKVYWQRQD----DGSFKIVYVEEKAIGTLIVTKAIsSN----  532
TGE    --YgGWQVLD-ATPQERSQGVFQCGPASVIgvreGDVgINFDMpFIFAEVNADRITWIYDNt---tGKQWkNSVnsHLIG:YISTKAVGSN----  430
TGP    --HDGWQVLD-STPQEISEGQFRIGPSPVSAIRQGLVQFMYDTTFVFTEVNGDKYIWLVKQNQERERRMSHRCGQCASIGKNISTKMVGEN----  416
B4.2   --YDGWQILDPSAPNGGGVLG-SCDLVPVRAVKEGTVGLTPAVSDLFAAINASCVVWKCCE----DGTLELTDSNTKYVGNNISTKGVGSD----  425
TGH    --YGGWQAVD-STPQETSEGVYQTGPASVLAVQRGEIGYMFDS2FVFSEVNADVVHWQEDDSSE-TGYKKL-KIDSYRVGRLLLTKKIGVDDDFG  506
Annu   AHYGGWQAVD-STPQELSDNMYRCGPAPVVAVKQGEVLRPYDSAYVFAEVNADKVFWRYSGP---TQPLKLIRKDMLGIGQNISTKAVGRF----  503

FXIII  --GMNDITDTYKFQEGQEEERLALETALMYG-AKKPLNTEGVMKS---------RSNVDMDFEVENAV-LGKDFKLSITFRNNSHNR-YTITAYL  553
TGC    --EREDITHTYKYPEGSsEERRAFTRAN-------HLNKLAeKEE----------TGmANRIRVGQSMNmGSDFDVFAHITNnTaEE-yvCRLLL  508
TGK    --MREDITYIYKHPEGSdAERKAVEtAAAHG----SKPNVYAnRgS---------AEDVAMQVEAQDAV-MGQDLmVSVmLiNhsSSR-RTVKLHL  611
TGE    --ARHDVTDKYKYPEGSdqERQVFQKALgKL----KPNtpFaATSSmglEtEeqEPSISGKIKVaGmLAVGKEVnLvLILKNIsrDt-KTVTvNH  518
TGP    --RRQDITLHYKFPEGSPEERKAMEKASGKR-PDDKLNSRTLHIS-------VLQNSVELGHPINLTI-VLKRKTATPQNVNIS------CSLDL  494
B4.2   --RCEDITQNYKYPEGSLGEKEVLERVEKEKMEREKDNGIRP-PS-------LETASPLYLLLKAPSSLPLRGDAQISVTLVNHSEQE-KAVQLAI  510
TGH    DADAEDITDQYKNKEGTDEERHSVLNAARSS----GFNYAFNLPS-------PEKEDVYFNLLDIEKIKIGQPFHVTVNIENQSSET-RRVSAVL  589
Annu   --QREDITNTYKYPEKSVEERAAMLKALRQS----ESLFSRYYLN-------EDFNDIHFNFELRDDIVIGSPFSVVVVMKNRSNQQDYTVTVLL  585

FXIII  SANITFYTGVPKAE---FKKETFDVTLEPL-SFKKEAVLIQAGEYMGQLL-EQASLHFFVTARINET---RDVLAKQKSTVLTIPEIIIKVRGTQ  640
TGC    CARTVSYNGILGPECG-TKyL-LNLTLePF-SEkSVPLcILYEKYrDCLT-ESNLIKVR--aLLVEPVINSYLLA-ERDIYLENPEIKIRILGEP  596
TGK    YLsVTfYTGVSGtifKETKKE---VeLAPGASDr-VtMPVAYKEYrPHLV-DQGAMLLNVSGHVKES---GQVLAKQHTFRLRTPDLSLTLLGAA  698
TGE    TAWTIiYNGTLVHEV--WKDS-ATmSLDPE-EEaehPiKIsYaQYERYLKsDNM-IRItAVCKVPDE---sEVVV-ErDiILDNPtLTLEVLneA  604
TGP    Q----TYTGNKKTNLGVIQKT---VQIQGQESEVSLSMDSSFYIYKLGMVDDEMVIKGFIIAEIVDS---GERVATDTTLCFLYSAFSVENPSTS  579
B4.2   CVQAVHYNGVLAAKL--WRXK-LHLTLSAN-LEKIITIGLFFSNFERNPP-ENTFLRLTAMATHSES---NLSCFA-QEDIAICRPHLAIKMPEKA  597
TGH    SASSIYYTGITGRK---IKRENGNFSLQPHQKEV-LSIEVTPDEYLEKLY-DYAMIKLYAIATVKET---QQTWSEEDDFHVEKPNLELEIRGNL  676
Annu   RVDTVLYTGHVKEG---VKKEKVERLTKAGAVEE-VRIDVSYEDYYKHLV-DQCAFNIACLATYHDT--NYEYFA-QDDFRVRKPDIKIKLEGEP  672

FXIII  VVGSDMTVTVQFTNPLKETLRNVWVHLDGPGVTRPM-KK-MFRE-IRPNSTVQWEEVCRPWVSG-HRKLIASMSSDSLRHVYGELDVQIQRRPSM  731
TGC    KQkRKLVAEVSLqNPLpVaLeGCTFTVEGAGLTEEQ-KtVEiPDPVEAGEEVKVRmDLvPlhmG-LHKLVVNFESDKLKAVKGfRNVIIGPA  686
TGK    VVGQECEVQIVFKNPLPvTLTNVVERLEGSGLQRP--KiLNVGD-IGGNETVTLRQsFVPVRPG-PRQLIASLDSPQLSQVHGVIQVDVAPapGd  789
TGE    rYRKPVNVQMIFSNPLDePVrdCVLmVEGSGIllgnlki-dvpt-lgPKErSRvRFdIIPsRSG-TKQLLADFSCNKFPaIKAMLsIDVaE  692
TGP    KVNQPLTITCNFKNTLPIPLTNIKESVESLGLNNM--KSWEQET-VPPGKTINFQIECTPVKTGNPRKFIVKFISRQVKEVHAEKVVLITK  667
B4.2   EQYQPLTASVSLQNSLDAPMEDCVISILGRGLIHRE-RSYRFRS-VWPENTMCAKFQFTPTHVG-LQRLTVEVDCNMFQNLTNYKSVTVVAPELS  689
TGH    QVGTEFVLAISLTNPLKRVLDNCFETIEAPGVTGAF-RV-TNRD-IQPEEVAVHTVRLIPQKPG-PRKIVATFSSRQLIQVVGSKQVEVLD  763
Annu   VQGQEMSAVATLKNPLPIPVKKGQELIEGPGIAKTQ-KIKLSCN-IAPCEEASVNFKFTPKYDG-RATIAAKFSSKELDDVDGFLNFNVEPKKEV  764

TGK    gGFISdAgGDShlGEtIPMAsRGGA                                                    814
B4.2   A                                                                            690
Annu   NCTGNAA                                                                      771
```

Fig. 2

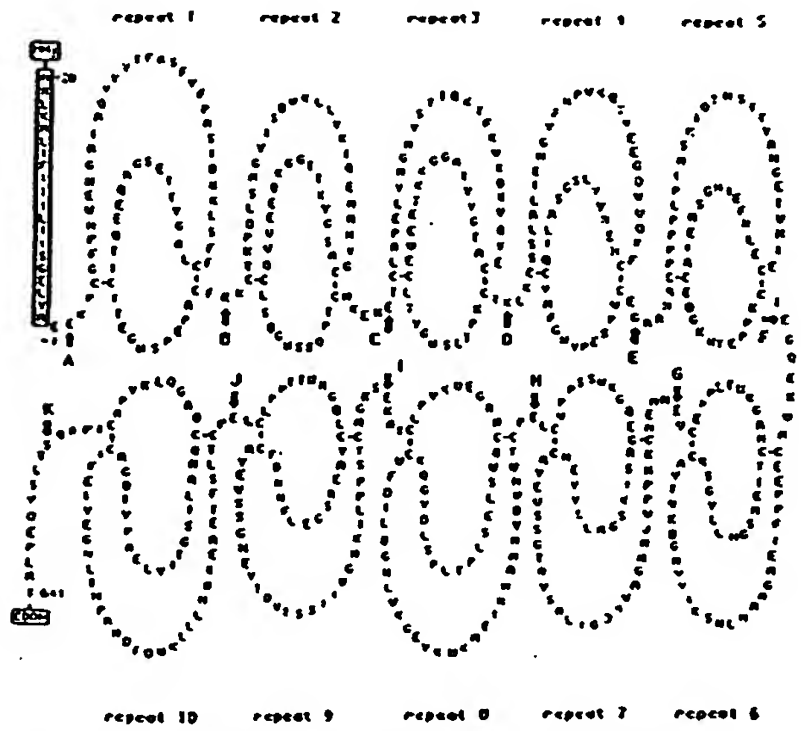

Fig. 3 Amino Acid Sequence of the b-Subunit of Human Factor XIII. The solid arrows indicate the location of introns (A–K) in the gene coding for the b-subunit. The signal sequence of 20 amino acids is enclosed in the long open box. The NH_2-terminal Glu residue in the mature protein is labeled 1, while the COOH-terminal Thr residue is labeled 641. (Reproduced from 20 with permission)

(32–35) and this is likely to occur via an "alternative" secretory pathway. A single transcript of 3.6–3.9 kb has been observed in varying amounts in different tissues: lung, heart, kidney, red blood cells > liver, spleen, testes; whereas it could not be detected in brain and thymus (23, 24, 26). However, the relative amount of protein in these tissues differs from the mRNA levels, and the protein is most abundant in liver and spleen, followed by heart, kidney and lung (Fig. 4 [36]). It appears that TG_C expression in different tissues is regulated not only by transcription but also on the translational level or by the rate of protein turnover.

3. Keratinocyte transglutaminase. The cDNA-cloning of TG_K from human and rat epidermal keratinocytes revealed a protein composed of 817/813 and 824 amino acids, respectively, with a molecular mass of ~89–90 kDa (37, 38). The proteins from rat and human display a high degree of sequence identity (92%). TG_K is peripherally associated to the cytoplasmic side of the plasma membrane through fatty acylation by thioester-linked palmitic and myristic acid (39, 40). A cluster of 5 cysteine residues in the NH_2-terminal extension (Fig. 2, res. 44–50) of TG_K are likely to provide the molecular basis for fatty acylation as is commonly observed in intracellular proteins. Indeed, deletion or conversion of the cysteine residues by site directed mutagenesis prevented membrane anchorage of the enzyme in transfection experiments (145) and a soluble form of TG_K results during terminal differentiation of keratinocytes *in vivo* from proteolytic processing of the 92-kDa to an ~80-kDa enzyme missing the membrane anchorage region (40). The NH_2-terminal extension of TG_K contains, in addition, a repeating 5-residue serine- and arginine-containing motif that is reminiscent of known phosphorylation sites for protein kinase C (37). Indeed, phorbol ester-stimulated phosphorylation of serine residues in the anchorage region of TG_K has been demonstrated (41). Phosphorylation might regulate the activity and/or substrate specificity of the membrane bound enzyme.

4. Epidermal transglutaminase. TG_E is a proenzyme that requires proteolytic activation and is the least understood of the "classical"

A

	Liver	Heart	Kidney	Lung	Spleen
specific activity (^{3}H cpm/mg protein)	876713.8	281944.0	219185.9	298581.8	454485.8
relative specific activity (%)	100	32.2	25.0	34.1	51.8

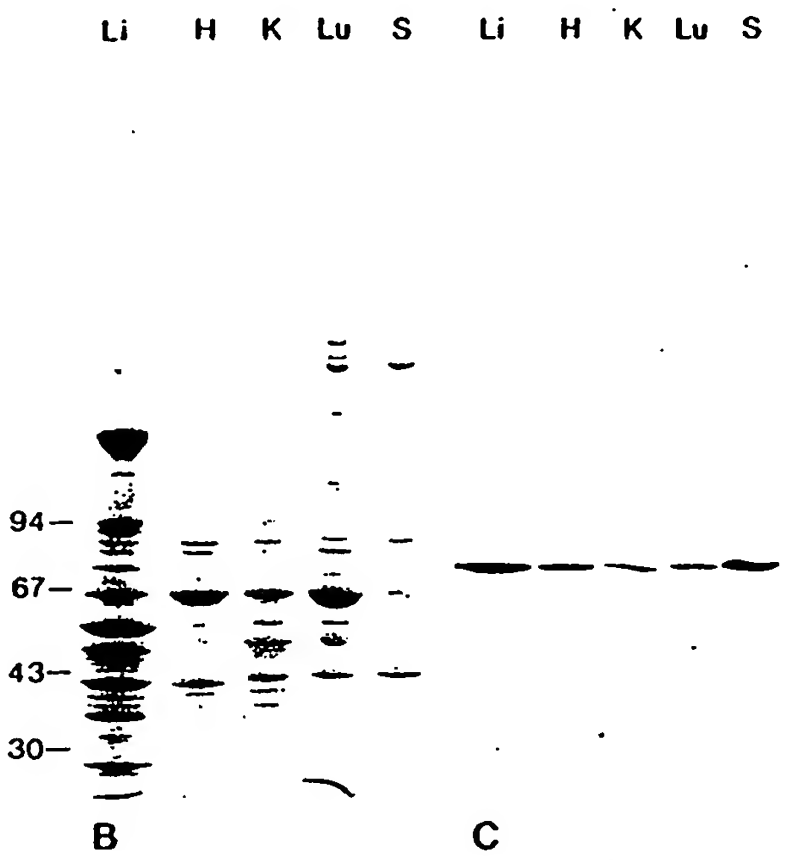

Fig. 4 Transglutaminase Activity and TG_C Protein Levels in Extracts of Various Guinea Pig Tissues. Tissues, liver (Li), heart (H), kidney (K), lung (Lu), and spleen (S) were extracted in 0.25 M sucrose (27) and the transglutaminase activity in the extracts (A) was determined by incorporation of [^{3}H]putrescine into *N,N*-dimethylcasein according to the standard protocol (32). The proteins in the different extracts were separated by SDS-PAGE in 3–15% polyacrylamide gradient gels and stained with Coomassie brilliant blue R (B) or after electrophoretic transfer to nitrocellulose by incubation with antibodies specific for TG_C (C [32]). M_r standards are indicated on the left

transglutaminases. The primary structure of TG_E was recently elucidated by cDNA-cloning from human and mouse epidermis and revealed a protein of 692 amino acids with a molecular mass of about 77 kDa (Fig. 2 [42]). The degree of sequence identity between species is low (only ~75% from mouse to human). Apparently, TG_E is still undergoing "rapid" evolution.

5. Prostate transglutaminase. Rat TG_P was reported to be a homodimeric protein of 150 kDa with monomers of 71 kDa by sedimentation equilibrium centrifugation or ~65 kDa by SDS-PAGE (43). Sequencing of cDNA-clones encoding rat TG_P showed a protein of 668 amino acids with a calculated M_r of 75'479 (Fig. 2 [44]). Although the protein is a major secretory product of rat dorsal prostate and coagulating gland (44), a signal peptide was not found in the deduced sequence and the protein was shown to be NH_2-terminally blocked, a typical feature of intracellular proteins (44). The presence of mannosyl residues and

Table 2 Amino acid identity between the different transglutaminase gene products. The extent of amino acid identity of factor XIII a-subunit (FXIII), tissue transglutaminase (TG_C), keratinocyte transglutaminase (TG_K), epidermal transglutaminase (TG_E), prostate transglutaminase (TG_P), band 4.2 (B4.2), hemocyte transglutaminase (TG_H) and annulin (Annu) was calculated from the sequence alignment (Fig. 2) in the region spanned by all gene products

	Annu	TG_H	B4.2	TG_P	TG_E	TG_K	TG_C
FXIII	34.1%	37.3%	26.0%	27.3%	34.2%	42.9%	36.3%
TG_C	35.7%	35.3%	33.1%	31.4%	37.0%	35.6%	–
TG_K	36.9%	40.0%	26.7%	35.4%	36.5%	–	
TG_E	33.1%	33.7%	30.9%	33.5%	–		
TG_P	31.7%	33.0%	27.2%	–			
B4.2	27.6%	26.5%	–				
TG_H	38.0%	–					

of a phosphatidylinositol anchor have recently been demonstrated (45). Moreover, secretion has been shown by immunogold electron microscopy to occur in apocrine secretory vesicles that are pinched off from the apical plasma membrane into the lumen where the contents are released after rupture of the surrounding membrane. TG_P appeared absent from endoplasmic reticulum and Golgi by this technique and is therefore likely to enter the vesicles directly from the cytoplasm (45–47).

6. Erythrocyte membrane protein band 4.2. Band 4.2 is a component of the cytoskeletal network underlying the erythrocyte plasma membrane. The complete amino acid sequence of human B4.2 has been deduced from its cDNA. It consists of 691 amino acids giving a protein of 77 kDa that shows a remarkable homology to known transglutaminases (Fig. 2, Table 2), but carries a cysteine → alanine substitution in the transglutaminase active site (48). Therefore, B4.2 represents a catalytically inactive member of the transglutaminase family. Membrane association is facilitated by myristylation of the penultimate glycine residue after cleavage of the terminal methionine (49, 50) and has been suggested to be stabilized by phosphorylation of B4.2 rendering an association-prone conformation (49). An isoform of B4.2 carrying a 30 amino acid insert after Gln-3 at the NH_2-terminus of the protein was identified and termed B4.2L (51). Moreover, a short form of B4.2 missing the sequence encoded by exon III (see below) has recently been described (49). This is the first time that alternative splicing has been shown to occur in the transglutaminase gene family. The difference in function of the isoforms is unknown, but B4.2L might be subjected to specialized posttranslational modification as has been observed in the NH_2-terminal part of other transglutaminase gene products.

7. Hemocyte transglutaminase and annulin, the arthropod analogues of vertebrate factor XIII and keratinocyte transglutaminase? Isolation and sequencing of the cDNA-clones coding for *Limulus* TG_H (52, 53) and the grasshopper protein annulin (54) showed proteins homologous to mammalian transglutaminases containing 764 and 772 amino acids with a molecular mass of 86 kDa and 87 kDa, respectively (Fig. 2). Both proteins contain neither a signal sequence nor N-linked glycosylation and disulfide-bonding, which would suggest that they are intracellular proteins (53, 54). In agreement, the NH_2-terminal amino acid of TG_H is posttranslationally modified (53).

Although annulin appears to be associated with the plasma membrane, it does not contain a transmembrane domain (54). TG_H and annulin do, for several reasons, appear to be most closely related to the a-subunit of factor XIII and TG_K. First, sequence comparison reveals that the extent of identity of mammalian factor XIII a-subunit and TG_K to TG_H and annulin is more pronounced than to *e. g.* TG_C or TG_P (Fig. 2, Table 2). This is a striking observation, considering the fact that TG_H and annulin are arthropod proteins, which separated from the vertebrate lineage early in evolution, and the other transglutaminases compared are mammalian proteins. Second, both TG_H and annulin contain NH_2-terminal extensions similar to those of the a-subunit of factor XIII and TG_K. The extension of annulin resembles the one of TG_K (37) in that it consists of two consecutive cysteine residues, potentially involved in fatty acylation for membrane anchorage, and the arginine- and serine-rich motif, thought to be a site for phosphorylation (Fig. 2). Third, TG_H is expressed in hemocytes and hepatopancreas (53) and fulfills a function similar to mammalian factor XIIIa in blood coagulation, while annulin shows a restricted expression in epithelial tissues (54) similar to TG_K. However, TG_H does not need proteolytic cleavage for activation.

Structure of Transglutaminase Genes

The genes for the a-subunit of factor XIII (55) and TG_K (56, 57) were shown to have 15 exons, whereas the gene for B4.2 (58) contains only 13 exons. However, with the exception of the first and the last exons which consist largely of noncoding sequence, and exon II of factor XIII and TG_K which encodes the nonhomologous NH_2-terminal extensions, all exons are of very similar or exactly the same size (Fig. 2, *triangles*). Exons X and XI of the factor XIII a-subunit and TG_K gene are fused to one exon in the B4.2 gene. This explains the smaller number of introns in the latter gene. In addition, there is not only a remarkable conservation of the positions of intron splice points but also of intron splice types. Considering the similarity in gene structure and the homology of the primary and predicted secondary structure, it is likely that the different transglutaminase genes are derived from a common ancestral gene. So far the human genome has been shown to contain a single gene copy per haploid genome for each type of transglutaminase characterized. Polymorphisms of both factor XIII a-subunit and TG_K have been observed in the human population indicating that the genes are present in different allelic forms (17, 55, 56). The genes for human factor XIII a-subunit, TG_K and B4.2 have been localized to chromosome 6 (20), 14 (56), and 15 (51), respectively. Thus, it appears that the transglutaminase genes became scattered in the genome after duplication of the ancestral gene. Despite the very similar

structure of the genes they differ remarkably in size. The gene for the factor XIII a-subunit is larger than 160 kb, whereas the genes for TG_K and B4.2 are only ~14 or ~20 kb, respectively.

The gene for band 4.2 differs in two respects from the other two genes of known structure. First, exon I and exon III of B4.2 can be alternatively spliced, thereby giving rise to the 30 amino acid larger B4.2L form that has been observed to be a minor component of reticulocyte membranes, and the shorter form observed in endothelial cells, respectively (49, 51, 58). Second, the B4.2 gene lacks the exon that encodes the NH_2-terminal extensions in the gene for factor XIII a-subunit and TG_K. This is consistent with a hypothetical divergence of the factor XIII a-subunit and TG_K from a lineage giving rise to TG_C, TG_E and B4.2 by gene duplication and subsequent addition of an NH_2-terminal extension (see below). The low degree of homology in the DNA sequence coding for the NH_2-terminal extension of TG_K from human and rat (37) indicates that this repetitive segment undergoes rapid change without influencing enzyme function. It appears probable that both the activation peptide of factor XIII a-subunit and the membrane anchorage region of TG_K evolved from an originally noncoding DNA segment by acquiring either the thrombin cleavage site or the cysteine cluster for membrane anchorage.

Structural Comparison of the Different Transglutaminases

Sequence comparison of the different transglutaminases within the region spanned by all gene products revealed that these enzymes are clearly related (Table 2). The sequences around the active site cysteines (Fig. 2, *cross*) show the most pronounced sequence homology. Although the sequences most highly conserved in TG_C from different species often correlate with the sequences most conserved between the members of the transglutaminase family (Fig. 2, *underlined*), this is not always the case (*e. g.* res. 118–132 of TG_C). These additional conserved regions might account for special properties of a single gene product. When sequences spanning the active site region of transglutaminases are used as probes in computer searches in protein databases (13) the catalytic regions of various thiol proteases are identified as the most closely homologous domains outside the actual group of transglutaminases. This indicates a distant structural relationship beyond the requirement for a catalytically active cysteine in both enzyme families.

Considering the rates of divergence of the transglutaminase genes between different species (0.085% amino acids changed per million year in TG_C from birds, rodents to human, and 0.116% and 0.045% amino acids changed per million year in TG_E and TG_K from rodents to human, respectively), it is likely that the vertebrate transglutaminases were distinct before reptiles separated from fish about 400 million years ago. This view is consistent with the observation that a human TG_K cDNA probe hybridizes to genomic DNA isolated from human, rat, chicken, toad and fish (57). However, the probe does not hybridize to *Drosophila* DNA (57), which might mean that divergence beyond a certain degree results in homology in only the minimal structural requirements for a functional transglutaminase.

A closer comparison of the different transglutaminase gene products reveals two evolutionary lineages (Fig. 5 [37]). B4.2 shows a clearly higher relationship to TG_C and TG_E than to any other gene product (Table 2). However, B4.2 appears to have rapidly diverged since separation from TG_C and TG_E, which is especially evident from the much lower degree of identity in the amino acids typically conserved among the different enzymes (Fig. 2, *underlined*). This might reflect the difference in function between B4.2, which has lost the catalytic activity, and the other transglutaminases. The similarity is most pronounced between TG_K and factor XIII a-subunit, indicating that this separation was the most recent in the evolution of the transglutaminases (Table 2). The origin of TG_P is unclear as it has diverged extensively and does not have a significantly higher sequence identity with any of the two lineages (Table 2). However, a comparison of distantly related proteins on the level of the primary structure is not always conclusive as the different gene products might have diverged at different rates and a final pedigree will require information about the gene structure of all enzymes.

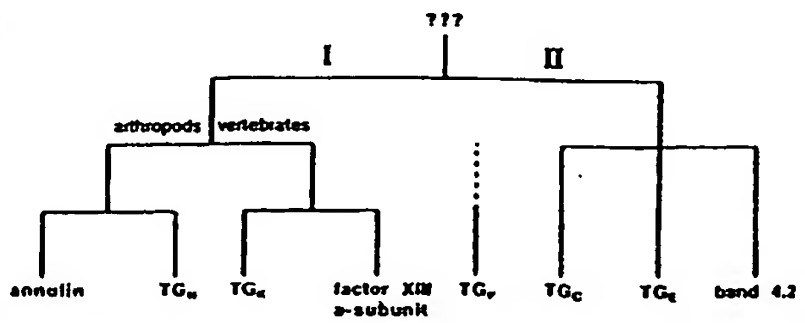

Fig. 5 Proposed Phylogenetic Tree of the Transglutaminase Gene Family. The proposed pedigree is based on the structural similarity (Fig. 2) and the sequence identity (Table 2) between the different gene products. A final classing of TG_P with the other transglutaminases was not possible with the presently available information

Regulation of Transglutaminase Expression by Signaling Molecules

1. Tissue transglutaminase. Retinoids have a wide range of developmental effects; retinoic acid has been shown to act as a morphogen in vertebrate limb development, and retinoids have been implicated in eye and epithelial development. TG_C expression was shown to be enhanced severalfold by retinoic acid *in vivo* and *in vitro*. The induction is due to higher mRNA (24, 59) as well as protein levels and occurs in many different cell types, *e. g.* in resident peritoneal macrophages (60), aortic endothelial cells (61), and hepatocytes (62). The induction occurs at physiological concentrations of retinoic acid (60) and leads to an intracellular accumulation of the enzyme (62, 63). The mediators of retinoic acid-induced TG_C expression were shown to be nuclear receptors for trans-retinoic acid, RAR-β and RAR-γ, as well as for cis-retinoic acid, RXR's. The expression of retinoic acid receptors and of the enzyme could be correlated in developing chick limb buds (64). Co-regulation of RAR-β and RAR-γ, but not RAR-α, with TG_C has been observed in many adult tissues, including trachea, lung, liver and bladder (65). The recent cloning of the promoter region of TG_C revealed that indeed consensus motifs of the estrogen receptor-type binding site are present which may function as retinoic acid responsive elements (66). The promoter region contained in addition presumptive responsive elements for interleukin-6 (66) and regulation of TG_C in hepatocytes by this cytokine on mRNA and protein level has recently been demonstrated (67). Interleukin-6 is involved in the regulation of the immune response, hemopoiesis and inflammatory reactions, and induces differentiation in certain cell-lineages, *e. g.* macrophages, and probably the concurrently occurring expression of the transglutaminase.

A similar induction of TG_C expression is observed by treatment of cells with the pharmacological agent sodium butyrate (63, 68). Butyric acid is believed to influence chromatin structure by inhibiting histone deacetylase and thereby causing a hyperacetylation of histones (for

review see 69). This produces a wide variety of effects on cells such as arrest of growth, promotion of differentiation, reversion of transformation characteristics and specific induction of various proteins. PC12 pheochromocytoma cells are able to differentiate either along the neuronal pathway into sympathetic-like neurons in presence of nerve growth factor or into cells showing the characteristics of mature chromaffin cells following treatment with butyric acid. Both differentiation promoters cause growth arrest, but only butyric acid-induced differentiation into chromaffin-like cells is accompanied by up-regulation of TG_C (68). Thus, TG_C expression strictly correlates with the differentiation of cells along certain maturation pathways.

Cells expressing high levels of the enzyme, due to induction with retinoic acid (61) or with sodium butyrate (68), or through transfection with multiple copies of the gene (63), show morphological features different from their normal counterparts. The cells change their appearance to a more spindle-like, flattened morphology, probably indicating an increased adhesion of the cells to the substratum. Interestingly, overexpression of the enzyme by induction *in vivo* (62) or transfection *in vitro* (63) does not lead to increased levels of cross-link formation. This indicates that the enzyme is largely present intracellularly in an inactive form and the activity of the enzyme is independently regulated from gene expression, perhaps by limited secretion.

The fusion of mouse alveolar macrophages, a process thought to resemble osteoclast formation, is induced by 1,25-dihydroxy vitamin D_3. The vitamin acts by up-regulating spermidine synthesis, which in turn leads to an accumulation of TG_C protein (70). Macrophage fusion can be separated into two steps, a first one involving spermidine dependent protein synthesis, and a second one comprising the actual fusion process which is strictly dependent on extracellular Ca^{2+} and transglutaminase activity. This indicates that TG_C might play a role in certain steps of the differentiation process of cells, rather than its up-regulation being a secondary effect.

2. Keratinocyte transglutaminase. TG_K plays a major role in the cross-linking of the cornified envelope during terminal differentiation of keratinocytes. There is increasing evidence for a regulation of keratinocyte differentiation by the phospholipase C and protein kinase C (PKC)-dependent signal transduction pathway, *i. e.* phosphoinositide turnover, intracellular [Ca^{2+}], and diacylglycerol. Phorbol esters and glucocorticoids were recently shown to induce TG_K on the transcriptional level in a PKC-dependent manner (71, 72), and the authors suggested that a specialized form of PKC, present mainly in lung and skin, might be involved. Studies on the promoter region of human and rabbit TG_K revealed a conserved AP1 site among other regulatory elements, *e. g.* SP1 and AP2 (72). Whereas no AP1 site was found in the TG_C promoter (66), other regulatory elements are present in both genes, supporting a unique role of this element in regulation of the TG_K gene. AP1 sites are known transcriptional regulatory elements for PKC activators such as phorbol esters. In addition, protein kinase C might also regulate TG_K on the protein level by phosphorylation in the membrane anchorage region (41). Retinoids suppress terminal differentiation of keratinocytes together with the expression of TG_K (72, 73) and of major cornified envelope substrate proteins (74, 75), whereas TG_C is expressed at high level (73). The coupled regulation of TG_K and the proteins forming the cornified envelope supports the notion that TG_K is the transglutaminase mainly involved in the cross-linking of this structure. Induction of transglutaminase activity by members of the TGF-β family had been described in cultured keratinocytes, without concurrently occurring squamous differentiation, and could be shown to be due to an up-regulation of TG_C, but not TG_K, at the transcriptional level (76). This relates to the physiological situation where TG_C is expressed by the undifferentiated basal keratinocytes.

3. Prostate transglutaminase. Androgen regulation of TG_P is demonstrated by its appearance in rats around the age of puberty and the decrease in synthesized protein following castration (43). The stimulation is due to increased transcription as shown by an approximately 80% decrease in mRNA subsequent to androgen withdrawal by castration and an increase to normal following treatment with the replacement testosterone (44).

Regulation of Transglutaminase Activity by Limited Proteolysis

Several transglutaminases are zymogens that need proteolysis for activation in addition to Ca^{2+}-binding.

1. Factor XIII. Activation of plasma factor XIII proenzyme (a_2b_2) to factor XIIIa (a'_2) is a two step process that is initiated by thrombin-catalyzed cleavage in the a-subunits at Arg-37 (Fig. 2) to yield the cleaved a'-subunits and the 37-amino acid activation peptides (13, 14), followed by Ca^{2+}-dependent dissociation of the b-subunits from the cleaved zymogen (77). The precise function of the b-subunit of factor XIII is not known, but it is thought to protect or stabilize the a-subunit or to regulate the activation of the zymogen in blood plasma. The intracellular form of factor XIII (a_2), *e. g.* from platelets, is activated by thrombin cleavage alone (77). A second specific cleavage site for thrombin-catalyzed proteolysis of factor XIIIa is located at Lys-513 (13). Cleavage at this site yields a 56-kDa fragment which contains the reactive thiol site and a 24-kDa carboxyl-terminal fragment. Contradictory results have been obtained about the effect of thrombin cleavage at this site on factor XIII activity (13, 15).

2. Epidermal transglutaminase. The zymogen form of TG_E is activated by proteolytic cleavage after Ser-469 (Fig. 2) into a 50-kDa fragment, containing the catalytically essential SH-group, and a 27-kDa carboxyl-terminal fragment (42, 78). The cleavage site corresponds to a ~9 amino acid insert unique to TG_E that is thought to form a flexible segment separating the two domains and that varies highly in composition between species (42). As the two domains remain noncovalently associated to each other, the cleavage is likely to induce a conformational change that leads to an exposure of the active site thiol. The activation of the zymogen is catalyzed by a number of different proteases including *e. g.* trypsin, thrombin, proteinase K and dispase (78). However, in the epidermis it is likely that the proenzyme is cleaved by a member of the Ca^{2+}-dependent cysteine proteinases, the calpains, which are known to be present in this tissue.

Regulation of Transglutaminase Activity by Divalent Cations and Nucleotides

Both reactions catalyzed by TG_C, the transfer reaction, leading to the incorporation of primary amines into peptide bound glutamine residues, and the esterolytic reaction, defined by the hydrolysis of p-nitrophenyl-acetate, depend on the same catalytic site in the protein (2, 4). Similarly, for both reactions an activation of the enzyme through a conformational change induced by binding of Ca^{2+}-ions is required. The enzyme was found to bind 3–4 Ca^{2+}-ions with a K_d of $1.0 \pm 0.6 \times 10^{-3}$ M by equilibrium dialysis (4). The metal ions bind to at least two sites on the enzyme with different affinities, thus giving rise to several possible enzyme-metal complexes. A complex containing metal bound to only one site catalyzes the esterolytic reaction, whereas the complex in which cations are bound at both sites acts in amine incorporation

into glutamine substrates (4). The specificity of cation-binding appears to be high as only Sr^{2+} can replace Ca^{2+} and activate, although at 10-fold higher concentration, but not Mg^{2+}, Ba^{2+}, or Mn^{2+} (4, 36). Several other metal ions such as Fe^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, etc., even strongly inhibit transglutaminase activity in presence of Ca^{2+} (4, 28). A similar cation dependence has been described for the other transglutaminases, *e. g.* factor XIII (3) and TG_K (79).

The inactivation of TG_C by Cu^{2+} has been shown to be due to an oxidation of free sulfhydryl groups to intramolecular disulfide bonds (28). Interchain disulfide bonds have not been observed. In contrast, Zn^{2+} has been shown to inhibit factor XIIIa in presence of Ca^{2+} in a competitive manner with a K_i of about 10^{-7} M (3). TG_C is similarly inhibited by Zn^{2+} (3), and the inhibition is accompanied by massive aggregation of the enzyme and is not due to disulfide-bond formation (D. Aeschlimann, unpublished results). Consequently, the inhibition of transglutaminases by Zn^{2+} occurs by a different molecular mechanism than inhibition by other heavy metal ions and the ratio of $[Zn^{2+}]/[Ca^{2+}]$ may play a role in regulation of transglutaminase activity. This might be important as Zn^{2+} shows an inverse intracellular to extracellular concentration relationship as compared to Ca^{2+} and may contribute to keep the intracellular enzyme in an inactive state.

Sequence analysis of the transglutaminase genes revealed no typical Ca^{2+}-binding motif, such as the EF-hand structure which comprises the calcium binding site of many other proteins, *e. g.* calmodulin. The Ca^{2+}-binding sites in transglutaminases are of low affinity and probably differ also in structure from the high affinity sites in typical Ca^{2+}-binding proteins. Regions rich in negatively charged residues were postulated to be potential Ca^{2+}-binding sites in transglutaminases (13, 24, 25, 38, 44).

Regulation of activity by guanosine nucleotides appears to be a characteristic feature of the tissue type enzyme (TG_C) independent of origin, whereas the activities of other transglutaminases such as factor XIIIa, TG_K, TG_p and TG_H remain unaffected (80). GTP and GDP, bind to TG_C in a reversible manner and give half maximal inhibition at ~100 µM and ~400 µM, respectively. In contrast, GMP and cyclic GMP show no inhibitory effects. Other nucleotides such as ATP or CTP inhibit transglutaminase activity first at a 1000-fold higher concentration. The binding of guanosine nucleotides and Ca^{2+}-ions is noncompetitive, although inhibition by GTP can be partially reversed by addition of Ca^{2+}. The inhibitory effect is due to conformational changes as demonstrated by the differential sensitivity of the enzyme to proteolytic digestion upon GTP- or Ca^{2+}-binding. This suggests that local concentrations of GTP and Ca^{2+} may regulate TG_C activity also *in vivo*. Inhibition of the enzyme by GTP is independent of the hydrolysis of the nucleotide as shown with nonhydrolyzable GTP-analogues, but the demonstration of GTPase activity ($K_{M[app]}$ ~4.4 µM) in the enzyme, might be of importance for its intracellular functions (81). However, sequence motifs conserved in the GTPase superfamily are not found in TG_C (23). It has recently been shown that TG_C with a cysteine → serine mutation in the active site retains the GTPase activity (82).

Physiological Functions of Transglutaminases

1. Factor XIII: Formation of the fibrin clot and wound healing. Factor XIII is the last zymogen to become activated in the coagulation cascade of vertebrates (for review see 3). It catalyzes the cross-linking of the fibrin γ-chains of different fibrin molecules to form the characteristic γ-chain dimers (83) followed by the covalent stabilization of α- and γ-chains (8, 84). These reactions are critical in blood coagulation as they result in the formation of an insoluble fibrin clot with defined physical properties. The activation of factor XIII by thrombin is promoted by fibrin ([for review see 15], 85). Factor XIII binds to the Aα- and Bβ-chains in the D-domain of fibrinogen with high affinity ($K_d = 10^{-7}$–10^{-9}M) and is thus associated with fibrinogen when circulating in blood plasma (85, 86). A second important reaction catalyzed by factor XIIIa is the cross-linking of α_2-plasmin inhibitor to the α-chain of fibrin, resulting in protection of the fibrin clot from degradation by plasmin (87). The cross-linking of fibrin and α_2-plasmin inhibitor occur at faster rate than that of other substrate proteins, thereby ensuring that these are the first reactions to occur (85, 87). Cross-linking of α_2-macroglobulin may similarly be important in protecting the blood clot from fibrinolytic reactions (88). Other substrate proteins for factor XIIIa such as fibronectin (9, 89), collagen (89), thrombospondin (90), vitronectin (91, 92), lipoprotein (a) (93), factor V (94), von Willebrand factor (95), and plasminogen-activator inhibitor-2 (96), are present in blood plasma and/or the vascular wall and may contribute to the stability of platelet-fibrin-endothelium association and to the wound healing process (97). Factor XIII binds to the surface of cultured fibroblasts (98) and mediates the cross-linking of cellular and plasma fibronectin at its NH_2-terminal matrix assembly site (99, 100). The increased rate of matrix assembly and the covalent stabilization observed in the presence of factor XIIIa may facilitate wound healing by providing an increased mechanical resistance to the wounded tissue. Accordingly, deficiencies of factor XIII or α_2-plasmin inhibitor lead to a "delayed" bleeding tendency although the primary hemostasis is normal. Cases reported include congenital deficiencies of the a- and/or b-subunit or acquired autoimmune response to factor XIII in disease of old age ([for review see 101] 102, 103).

2. Tissue transglutaminase: Stabilization of extracellular matrices and formation of cross-linked cell envelopes in programmed cell death. TG_C was the first transglutaminase described (36) and is present in substantial amounts with a broad tissue distribution in vertebrates (32, 33, 104). Even so, its physiological function remains uncertain. Two main functions have been proposed which take place in different compartments and would require the presence of TG_C in both the extracellular space and in the cytosol.

There is increasing evidence for TG_C activity in the extracellular space. Here, the Ca^{2+}-concentration is high enough for enzyme activation and inhibitory factors such as GTP or Zn^{2+} are largely absent. A number of extracellular proteins like fibrin(ogen) (8, 86), fibronectin (9, 105), vitronectin (91, 92), nidogen/entactin (7, 32), collagen type III N-propeptide (106), collagen type II, osteonectin/BM-40/SPARC (33) and osteopontin/SPP-1 (107) have been shown to be specific glutaminyl substrates for TG_C. It is, however, not clear if all these potential substrates are cross-linked by TG_C *in vivo*. Fibrinogen and fibronectin have been shown to bind to the surface of hepatocytes and endothelial cells in suspension culture and become cross-linked into the pericellular matrix. This process is mediated by TG_C as shown by inhibition with TG_C specific antibodies and the formation of fibrinogen Aα-chain polymers characteristic for TG_C action (34, 108). In agreement, a large, fibronectin-containing insoluble protein polymer associated with plasma membranes in liver was shown to be formed by the action of a transglutaminase (109). TG_C covalently stabilizes laminin-nidogen complexes in the homoaggregate structure thought to occur in basement membranes and does often co-localize in the extracellular space with the laminin-nidogen complex and with fibronectin (32). In the development of long bones, TG_C is externalized from hypertrophic chondrocytes and participates in matrix cross-linking before the tissue undergoes calcification (33). TG_C may modulate cell-matrix interactions (97), thereby facilitating the assembly of the matrix, and play a role in events related to wound healing and excessive tissue repair, such

as the formation of the highly insoluble matrix found in cirrhotic liver (110). It has been shown that TG_C binds to the extracellular matrix with high affinity after wounding of cells (111), modulates the biological activity of interleukin-2 in regeneration processes (112), and regulates the conversion of latent into active TGF-β (35), perhaps through increasing plasmin activity by cross-linking molecules belonging to the plasminogen-activator inhibitor class (96). Even though the sum of these observations strongly indicates an extracellular function for TG_C, a proper understanding is first possible when the "secretory" mechanism for this and other transglutaminases has been elucidated.

The process of programmed cell death, also called apoptosis, plays a role in a number of fundamental processes such as metamorphosis, embryonic morphogenesis, and hormone-induced tissue remodelling. Transglutaminases are known to be involved in apoptosis and the role of TG_C in *e. g.* the cross-linking of plasma membrane and cytoskeletal components in terminal steps of erythrocyte maturation is well established (for review see 3). It appears that the observed change in cell morphology results from cross-linking of intracellular proteins like band 3, band 4.1, spectrin and ankyrin, after activation of the enzyme inside the cell. While band 3 is the major amine acceptor in the erythrocyte membrane, other intracellular substrates have been described in other systems and may participate in cross-linking in apoptotic cells, *e. g.* actin (113) and lipocortin I/annexin I (114). It is likely that Ca^{2+} influx into the cytoplasm, either from intracellular Ca^{2+} storage compartments as observed in terminal differentiation of keratinocytes (115), or from the extracellular space as observed in terminal maturation of erythrocytes (3), activates the enzyme. A role for TG_C in the apoptotic program of other cells has been postulated. It is accumulated in the cytoplasm of hepatocytes undergoing terminal differentiation both *in vivo* and *in vitro* ([for review see 116] 62, 117).

TG_C has also been implicated in pathological events. The loss of transparency in ageing eye lens, leading in the final stage to the senile cataract, is in part due to increased levels of γ-glutamyl-ε-lysine cross-links (3). Endogenous TG_C of the eye lens cortex has been shown to be responsible by specifically cross-linking β-crystallins (118–120). Increased cross-linking in the vascular walls due to hemolysis or endothelial destruction may promote the formation of artherosclerotic plaques (121). Phospholipase A_2 (PLA_2) may play a pathogenic role in inflammatory diseases such as rheumatoid arthritis. TG_C has recently been reported to be expressed at high level in rheumatoid, but not osteoarthritic lesions (122) and to activate PLA_2 by a conformational change, induced by intramolecular cross-linking, that stimulates PLA_2 dimerization (123).

3. Keratinocyte and epidermal transglutaminase: Terminal differentiation of epithelia and formation of the cornified envelope in the epidermis. When epidermal cells undergo terminal differentiation several proteins become cross-linked to form the cornified envelope, a 10–20 nm thick deposit of protein on the intracellular surface of the plasma membrane (for review see 115). The cross-linking is due to disulfide-bonding of *e. g.* keratins and to transglutaminase cross-links. Transglutaminases are activated by influx of Ca^{2+} into the cytoplasm when the cellular membranes loose their integrity during the final maturation steps (115). Several transglutaminase substrates such as keratolinin (124), involucrin (125, 126), cornifin (75), and sciellin (127) appear to be covalently incorporated into the cornified envelopes, but so far only loricrin has been actually shown to be cross-linked to the cornified envelopes by γ-glutamyl-ε-lysine bonds (74). Cross-linking of cysteine and serine protease inhibitors such as cystatins (128, 129) or SKALP/elafin (130) might finally protect the cornified envelopes from proteolytic attack. Three different transglutaminase transcripts of 2.9 kb, 3.3 kb and 3.7 kb have been observed in epidermis (38). The smallest is coding for TG_K, the largest for TG_C, and the medium sized represents TG_E (38, 42, 73, 131). The membrane bound TG_K co-localizes with the identified substrate proteins within the supra-basal layers of epidermis, whereas TG_C expression is restricted to the basal keratinocytes (73, 75, 126, 131). It appears likely that TG_K is the transglutaminase predominantly involved in squamous differentiation of epithelia (15, 131) as it is the most abundant transglutaminase in this tissue (42), although it is also present in epithelia that do not normally keratinize such as in oral cavity, oesophagus, trachea, lung, liver and intestine (72, 79, 129). The soluble TG_E is found in advanced stages of terminal differentiation of epidermal cells and in the embryologically related hair follicle cells (42, 78, 132). It might play a role in hair shaft formation by cross-linking of *e. g.* trichohyalin (133), but is also likely to contribute to the formation of the cornified envelope in the later stages of epidermal differentiation. An additional transglutaminase with a subunit structure identical to that of TG_E has been identified in hair follicles based on its unique charge properties (132). It is likely that this enzyme is a variant form of TG_E which has undergone different posttranslational modifications, but the possibility remains that the hair follicle enzyme is a distinct gene product. The differential distribution of transglutaminase isoenzymes with varying substrate specificity in epidermis indicates that regulation of protein cross-linking is essential for the production of mature cornified envelopes and hair shafts.

4. Prostate transglutaminase. Formation of the copulatory plug. In rodents, such as rat or guinea pig, rapid coagulation of the seminal fluid is responsible for the formation of the copulatory plugs that are highly enriched in γ-glutamyl-ε-lysine cross-links (for review see 134). The clotting results from cross-linking of proteins derived from the seminal vesicles such as guinea pig SVP-I (135) or rat SVP-IV (136) by an enzyme, most likely TG_P, secreted by the dorsal prostate and coagulating gland (44). TG_P constitutes up to 25% of total intracellular protein in these tissues (43, 45). The cross-linking of seminal vesicle proteins may be competed for by polyamines, *e. g.* spermine and spermidine, which are produced in excess by the ventral prostate of certain species. Present in excess, these low molecular weight amines prevent a premature coagulation of semen during its passage through the distal part of urethra (134).

5. Band 4.2 protein: The plasma membrane cytoskeletal network. Band 4.2 protein is a structural component of the cytoskeletal network underlying the erythrocyte plasma membrane, but is also found in other cells and tissues such as platelets, kidney and brain. It associates with the cytoplasmic domain of the anion exchanger band 3 and possibly interacts also with ankyrin and band 4.1 protein (for review see 49). Thus, it is likely that B4.2 still retains the transglutaminase protein-binding site but is unable to covalently cross-link the bound proteins. This points to the possibility of a role of transglutaminases as structural components, beside functions involving the catalytic activity. Interestingly, the second transglutaminase present in erythrocytes, TG_C, catalyzes the covalent stabilization of erythrocyte membrane cytoskeletal proteins, *e. g.* band 3, in ageing erythrocytes (3). This poses the question if B4.2 and TG_C could compete for the same binding sites on proteins and one function of B4.2 might be to prevent cross-linking of the cytoskeletal network in intact cells. Furthermore, the recent association of the murine mutation *pallid* with the B4.2 gene indicates an essential role for B4.2 in cellular organelle membrane integrity and function (49, 137). This mutation is classified with the platelet storage pool deficiencies and affected animals suffer in a prolonged bleeding time, reduced kidney lysosomal enzyme secretion and aberrant pigmentation due to a defect in secretory vesicle maturation or release.

6. Invertebrate transglutaminases: Proteins involved in hemolymph coagulation and morphogenesis in arthropods. The arthropod hemolymph coagulation cascade resembles the vertebrate blood coagulation system in that it is propagated by the activation of several serine protease zymogens and results in the conversion of the soluble coagulogen, a fibrinogen-related molecule, into the insoluble fibrillar coagulin network. The zymogens and coagulogen are contained within cytoplasmic granules in hemocytes, circulating in the hemolymph, and are released by exocytosis upon triggering by bacterial endotoxins (lipopolysaccharide). The resulting clot is cross-linked by γ-glutamyl-ε-lysine cross-links by a transglutaminase (2), designated hemocyte transglutaminase according to its origin (52, 53). Even though it remains to be established whether coagulin itself is cross-linked within the clot, other cross-linked components have been identified (52).

Annulin expression in the insect embryo occurs in areas undergoing morphogenetic rearrangements (54). Annulin is expressed in narrow circumferential bands of epithelial cells at the boundaries of developing limb segments and precedes the first morphological signs of segmentation. Its potential role during development could be to stabilize or modify the membrane and subcortical structures of cells that undergo morphogenetic shape changes and are exposed to mechanical stress.

Catabolism of the γ-Glutamyl-ε-Lysine Isodipeptide Bond

There is no evidence that γ-glutamyl-ε-lysine cross-links are cleaved without prior degradation of the proteins. The dipeptide released by proteolytic degradation of cross-linked proteins appears to be catabolized most efficiently in kidney. The reaction (EC 2.3.2.4) that is catalyzed by the enzyme γ-glutamylamine cyclotransferase proceeds by a cyclic transfer with production of pyroglutamic acid and lysine (138). Different isoenzymes with distinct specificities exist, one of which exhibits reactivity towards only ε-(L-γ-glutamyl)-L-lysine, but not α-(L-γ-glutamyl)-L-lysine.

Conclusions and Perspectives

Members of the transglutaminase family, *e. g.* the a-subunit of factor XIII and TG_P, are present and biologically active in the extracellular compartment of the vertebrate body, although they are not conventionally secreted through the endoplasmic reticulum-Golgi complex. Also TG_C occurs in the extracellular space (32, 33) and appears to play a role in *e. g.* matrix remodelling during terminal differentiation of cartilage (33). A not yet resolved key question in transglutaminase biology is the mechanism(s) that leads to the externalization of these enzymes. This route may be similar to that of other proteins that do not follow the conventional secretory pathway, such as acidic and basic fibroblast growth factor (FGF), interleukin (IL)-1α and 1β, platelet-derived endothelial cell growth factor (PD-ECGF), ciliary-neurotrophic-factor, lymphocyte maturation factors designated thymosins, an adult-T cell-derived factor (ADF) belonging to the thioredoxin protein family, the lactose-binding lectins L-14 and L-29, and annexins/lipocortins (139–143 and ref. therein). These proteins have in common that the milieu in the endoplasmic reticulum could interfere with structures essential for their biological function. The oxidizing environment in this compartment could potentially inactivate transglutaminases, ADF, the lectin L-14, IL-1β, basic FGF and PD-ECGF that depend on free sulfhydryls for activity or adopt inactive conformations upon disulfide-bond formation (140, 141, 143). Alternatively, the high Ca^{2+}-concentrations in the endoplasmic reticulum could activate the transglutaminases and lead to inappropriate protein cross-linking. The presence of cytokines and their receptors in the same compartment could lead to autocrine stimulation and transformation of the cell. Indeed, several oncogenes turned out to encode FGF-related sequences fused to a hydrophobic leader sequence (for review see 139, 143).

Evidence for several different mechanisms for externalization has been presented in the literature. It was suggested that temporary disruptions of the plasma membrane in cells subjected to mechanical stress could be a release mechanism, and it was demonstrated that a growth promoting activity, likely to be basic FGF, is released by mechanically wounded, but not by metabolically poisoned endothelial cells (144). Vesiculation has been described in several biological systems, *e. g.* in the intestinal brush border, in mineralizing cartilage, in maturation of erythrocytes and in ageing of hepatocytes, and it has been shown that vesicular release is responsible for the externalization of IL-1β and a lectin expressed by differentiating myoblasts (140, 141). It has been suggested that N-acylation could be one signal for sequestration of cytosolic proteins into "secretory" vesicles as many of these proteins, including TG_C and the a-subunit of factor XIII, are NH_2-terminally acetylated (31). Indeed, release by membrane "blebbing" has recently been demonstrated for TG_P in apocrine secretion of prostate (45–47). A superfamily of transport ATPases, referred to as the mammalian multidrug resistance P-glycoprotein and related molecules, has recently been discovered. Members of this protein family facilitate the transport of *e. g.* ions, sugars, amino acids, and peptides across membranes in different organisms and could act in concert with chaperons in facilitating membrane transport of "small simple" proteins (for review see 139). Release of transglutaminases by necrotic cell lysis cannot be excluded, but appears unlikely as the sequestering cells, *e. g.* chondrocytes, often appear morphologically intact and are active in protein synthesis (33; D. Aeschlimann, unpublished results). Similar evidence excluding cell death as the major mechanism for release has also been obtained for other proteins with an uncertain secretory pathway (140, 141, 143). Unconventionally secreted proteins, including TG_C, TG_P and factor XIII a-subunit, generally appear to be present in high concentrations in the cell cytosol. A high level of synthesis is not necessarily coupled to externalization and secretion appears to be specific for the cell type or differentiation stage (33, 140, 142). Overexpression of TG_C in fibroblasts does not lead to increased cross-link formation (63), indicating that the intracellular enzyme is inactive and that activation of TG_C is regulated independently from its biosynthesis, possibly by limited externalization. It is tempting to speculate that an alternative secretory mechanism does exist, is actively regulated by the cell, and enables the access of certain transglutaminases to the extracellular space.

Although it is clear that the specificity in transglutaminase-catalyzed cross-linking of proteins at least in part resides in the sequences in the vicinity of substrate glutamine residues (5, 6), it has not been possible to derive a consensus sequence from the glutaminyl substrate sites determined in a number of different proteins (for review see 7). This might be explained by a considerable dependence on the conformation of adjacent regions of the substrate protein. Studies on the specificity of factor XIIIa and TG_C for the amine acceptor sites in β-casein (5, 6) and fibrin(ogen) (8) demonstrate that transglutaminases differ in their binding affinity and/or the catalytic rate for a particular substrate site/protein, although they often also cross-link at the same sites, at least under *in vitro* conditions. Thus, in a tissue where a series of different substrate proteins compete for a transglutaminase, the affinity of the interaction between the enzyme and a particular substrate protein is

important. It appears essential to determine not only the amine acceptor glutamine residue(s) after amine incorporation *in vitro*, but also to assess the affinity for the interaction of the transglutaminase with the substrate protein in the presence of physiologically occurring alternative substrates. It is not unlikely that the cross-linking site is not the only site in a substrate protein that interacts with the transglutaminase and it is possible that the different determinants for enzyme recognition are held in the correct position in the secondary and/or tertiary structure (7, 33). TG_C has been shown to bind strongly to certain extracellular matrix proteins, through an interaction that can only be dissociated by use of chaotropic salts (33, 86, 105, 111). The ability of transglutaminases to bind tightly to certain proteins and to self-aggregate may have biological functions other than in protein cross-linking as indicated by the fact that a catalytically inactive transglutaminase, B4.2, functions as a link in the erythrocyte membrane cytoskeletal network.

It might also be important to determine the lysine residue(s) that are cross-linked to a certain glutamine residue in the tissue. This will be technically difficult and laborious, as many different lysine residues of a protein, or even of different proteins, in the tissue might compete for the same glutamine residue, leading to the formation of extremely heterogeneous oligomeric structures, at least when a protein is cross-linked by a transglutaminase *in vitro* (2, 136). Even though the specificity is poor, in the few cases where the physiologically cross-linked lysine residues have been determined, a clear preference for certain lysines has been observed. In the fibrin clot, the γ-chains are reciprocally cross-linked in the overlapping antiparallel carboxyl-terminal segments at the same glutamine and lysine residue in each chain (83). Three cross-linked peptides were isolated from proteolytic digests of cornified envelopes of skin and shown by sequence analysis to originate from loricrin molecules with heterogeneous modifications involving 3 out of 14 glutamine and 2 out of 7 lysine residues (74). The glutamine residues involved in cross-linking of crystallins in the ageing eye lens are exclusively located in the NH_2-terminal extensions of β-crystallins, apparently important for self-aggregation of these proteins (118). A single carboxyl-terminal lysine in αB-crystallin has recently been identified as a major amine donor in this tissue (119, 120). It appears likely that the structure of the protein assembly rather than the protein components itself determine the location of the cross-linking, although certain structural elements are required. Elucidation of the protein sites involved in cross-linking *in vivo* might provide an insight into the sterical arrangement of the protein assembly in cells and extracellular matrices.

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Appendix B

Transglutaminase Autoantibodies in Dermatitis Herpetiformis and Celiac Sprue

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Dermatitis herpetiformis (DH) is an autoimmune blistering skin disorder that is associated with intestinal gluten sensitivity. Epidermal transglutaminase (TGe) and closely related tissue transglutaminase (tTG) are considered to be autoantigens in DH, because a majority of DH patients have IgA specific for TGe and for tTG. It is unknown where and how these autoantigen-specific IgAs are generated in DH. Results reported in this paper on nine DH patients on a gluten containing diet demonstrate that the levels of circulating anti-tTG IgA and anti-TGe IgA in DH are correlated with each other and that both appear to be correlated with the degree (extent) of enteropathy. An analysis of 15 untreated celiac sprue (CS) patients demonstrated that approximately 33% of CS patients had elevated levels of anti-TGe IgA. These results would indicate that intestinal damage is associated with the production of anti-tTG IgA and anti-TGe IgA in DH patients.

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INTRODUCTION

Dermatitis herpetiformis (DH) is an autoimmune, blistering, intensely pruritic papulovesicular rash typically located on the elbows, forearms, buttocks, knees, and scalp (Hall, 1992; Fry, 2002). It is often associated with an enteropathy characterized by villous atrophy and/or increased infiltration of intraepithelial lymphocytes (Fry, 1995; Cooney *et al.*, 1977). The enteropathy and the rash are caused by the ingestion of gluten, which is a group of storage proteins of wheat, barely, and rye. Another gluten sensitive disease, celiac sprue (CS), results from a potent inflammatory response to gluten within the small intestine (Reunala, 1998). Additionally, even though gluten is the exogenous antigen for both CS and DH, these two diseases have characteristics of autoimmune disorders. These characteristics include a tight association with major histocompatibility complex II molecules (HLA-DQ2 and HLA-DQ8) and the production of circulating autoantibodies (Spurkland *et al.*, 1997; Sollid, 2000).

In 1984, Chorelezki reported that autoantibodies directed against endomysial tissue were present in both DH and CS and could be used as a marker for both diseases (Chorzelski *et al.*, 1984). Subsequently, it was found that these endomysium-binding autoantibodies were directed specifically against tissue transglutaminase (tTG) (Dieterich *et al.*, 1997; Dieterich *et al.*, 1999; Porter *et al.*, 1999). The presence of circulating anti-tTG IgA is commonly used as a screening tool for celiac disease (James, 2005). Anti-tTG IgA antibodies are also diagnostic markers for enteropathy in DH patients (Kumar *et al.*, 2001). Thus, anti-tTG IgA is an autoantibody that is found in both diseases.

Interestingly, DH patients with villous atrophy have high levels of circulating anti-endomysial IgA (Volta *et al.*, 1992). Levels of circulating anti-tTG IgA and anti-endomysial IgA are correlated with the absence or presence of enteropathy in CS patients, suggesting that these antibodies are produced in the setting of mucosal injury (Kotze *et al.*, 2003; Rostami *et al.*, 2003). Yet another study demonstrated that IgA deposits and tTG colocalize in the jejunal samples of celiac patients and that this IgA was tTG specific based on binding studies of eluted jejunal IgA (Korponay-Szabo *et al.*, 2004).

In 2002, another autoantigen, epidermal transglutaminase (TGe), was identified for DH (Sardy *et al.*, 2002; Karpati, 2004). In the perilesional tissue of DH patients, the IgA deposits at the dermal/epidermal junction were found to colocalize with TGe in the papillary dermis and small vessels (Karpati, 2004; Preisz *et al.*, 2005). DH patients also had circulating TGe-specific IgA that fell into two groups. One antibody group bound to TGe exclusively, whereas the second antibody group was crossreactive and bound to both tTG and TGe. This second group was found in celiac patients as well and had a lower avidity for TGe than the first group. The level of circulating TGe-specific IgA was lower in DH patients on a gluten-free diet. This suggests that anti-TGe IgA is also dependent upon the continued intestinal exposure to gluten in DH patients, similar to anti-tTG IgA in CS patients.

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Abbreviations: CS, celiac sprue; DH, dermatitis herpetiformis; tTG, tissue transglutaminase; TGe, epidermal transglutaminase

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It is of interest then, as to whether the levels of anti-TGe IgA or anti-tTG IgA that are found in DH or CS patients are related to the degree or extent of mucosal injury, such as villous atrophy, as a result of intestinal exposure to gluten. To determine whether there is a correlation between the levels of circulating anti-TGe IgA and enteropathy/villous atrophy in DH and CS patients, we compared the titers of anti-TGe IgA and anti-tTG IgA with the presence and severity of villous atrophy.

RESULTS

Characterization of DH and CS patients

Nine DH patients that had not been on a gluten-free diet were evaluated for this study (Table 1). Duration of blistering disease varied from less than 0.5 years to 9 years (Table 1). Fifteen untreated celiac patients who had never developed a pruritic rash were evaluated (Table 2).

Correlation between the levels of anti-TGe IgA and anti-tTG IgA in DH and CS patients

The levels of anti-TGe IgA and anti-tTG IgA were highly correlated in DH patients, with a Pearson's coefficient, $r=0.74$. (Figure 1a). There was no significant correlation between the two autoantibodies in the untreated celiac patients ($r=0.286$, Figure 1b).

Relationship between enteropathy and serology

Marsh Scores were determined from the histopathological analysis of the duodenal biopsies of six of the nine DH patients and all of the 15 celiac patients. The scores varied from 0 to 3c (Tables 1 and 2). All biopsies were evaluated using the Marsh system of scoring (Oberhuber, 2000).

With the DH patients, there was a significant correlation between the levels of anti-TGe IgA and the degree of enteropathy ($r=0.82$) (Figure 2a). A significant correlation was also found between the marsh scores and levels of anti-tTG IgA ($r=0.7$) (Figure 2b). There was no correlation between the levels of anti-TGe IgA and the level of villous atrophy in the celiac patients (Figure 2a). Anti-tTG IgA also does not correlate with the degree of villous atrophy in CS patients (Figure 2b). However, all 15 celiac patients had elevated levels of anti-tTG IgA, thus supporting previous reports that elevated levels of anti-tTG IgA do correlate with the presence of enteropathy.

DISCUSSION

Several lines of evidence support TGe as a target for IgA autoantibodies in DH. Sardy *et al.* (2002) demonstrated that the IgA deposits in the perilesional skin of DH patients colocalizes with TGe, that DH and CS patients had circulating anti-TGe IgA in their blood, and that DH and CS patients on a gluten-free diet had lower levels of anti-TGe IgA than those that were not. One possible explanation for this is that the enteropathic process leads to the production of circulating anti-TGe IgA in both CS and DH patients.

Our results suggest a correlation among the extent of enteropathy, anti-tTG IgA and anti-TGe IgA in DH patients. Albeit, greater numbers of DH patients that are on a gluten containing diet would be necessary for definite proof of this correlation. This result would suggest that the production of these antibodies occurs as a result of mucosal damage in the intestine of DH patients. This would certainly fit the accepted theory that all DH patients have some level of intestinal immunopathological response to gluten that manifests as a skin condition.

Another important conclusion to be made from these results is that some celiac patients produce anti-TGe IgA, which supports a previous finding (Sardy *et al.*, 2002). The production of this antibody in celiac patients, however, did not appear to be correlated with severity of villous atrophy in their intestine. Similarly, the production of anti-tTG IgA in celiac patients did not correlate with the severity of enteropathy, which would also support a previous finding (Tursi *et al.*, 2003). One contributing factor to this lack of correlation between the production of anti-tTG and anti-TGe antibodies and the severity of enteropathy may be the fact that CS patients on a gluten-containing diet by definition have Marsh scores of 3 or greater, whereas all levels of severity can be present in DH patients on a gluten containing diet. Overall, however, these results would indicate that the

Table 1. Evaluated DH patients

DH patient no.	Age (years)	Sex	Disease duration (years)[1]	Marsh score	Villous atrophy
1	48	F	<0.5	NA	?
2	75	F	<0.5	0	No
3	57	M	5	0	No
4	68	M	9	NA	?
5	70	F	<0.5	3c	Yes
6	65	F	<0.5	NA	?
7	35	M	<0.5	3b	Yes
8	70	M	<0.5	3b	Yes
9	75	M	3	1	No

F, female; M, male; NA, not applicable.
[1]All DH patients had IgA deposits in perilesional tissue.

Table 2. Evaluated CS patients

CS Patient no.	Sex	Age	Disease duration (years)	Marsh score	Villous atrophy
1	M	13	0.5	3c	Yes
2	F	71	0	3b	Yes
3	F	64	0	3c	Yes
4	M	58	0.5	3b	Yes
5	F	48	0	3c	Yes
6	F	32	0	3c	Yes
7	F	64	0	3c	Yes
8	F	37	0	3c	Yes
9	F	40	0	3c	Yes
10	F	58	0.5	3b	Yes
11	M	65	0.25	3b	Yes
12	M	39	0	3a	Yes
13	F	50	0	3a	Yes
14	F	61	0	3c	Yes
15	M	52	0.25	3a	Yes

F, female; M, male.

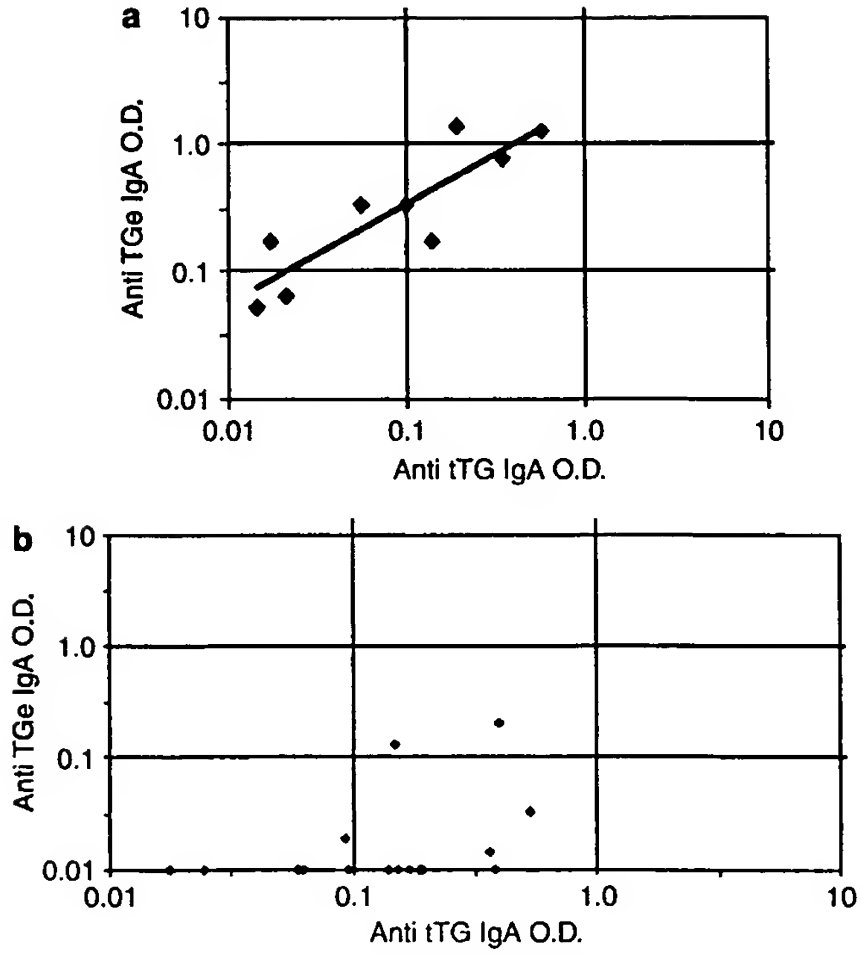

Figure 1. Correlation between anti-tTG IgA and anti-TGe IgA levels in the sera of DH and CS patients on a normal gluten containing diet. OD values for anti-TGe IgA were plotted along the *y* axis and corresponding anti-tTG IgA values for each patient plotted along the *x* axis. All patients were on a normal gluten-containing diet. Nine DH patients were evaluated (a) (Pearson's coefficient $r=0.74$), as well as 15 CS patients (b) ($r=0.286$).

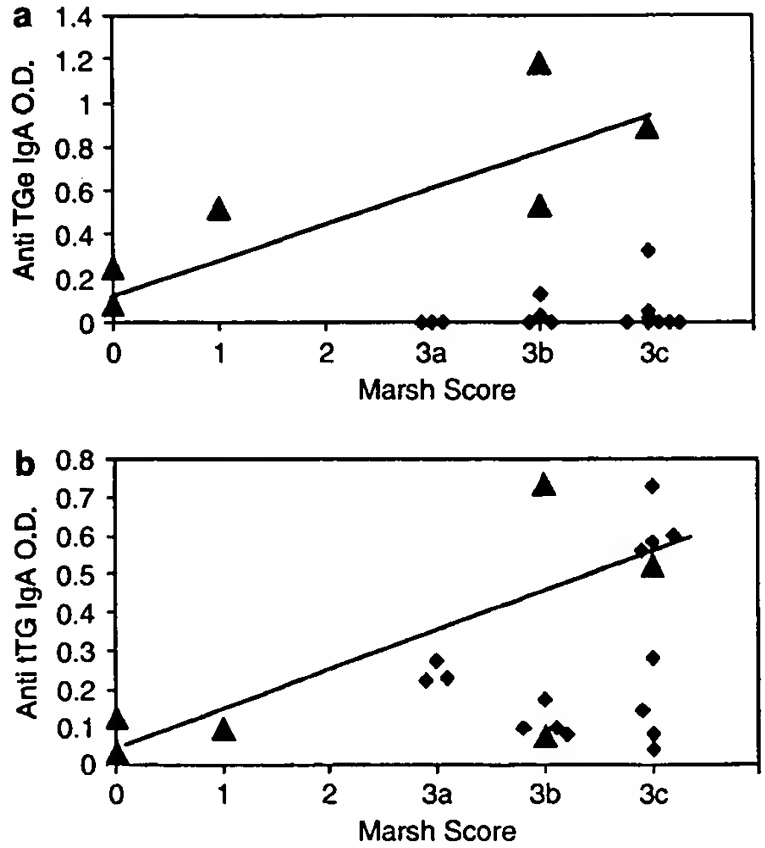

Figure 2. Comparing Marsh Scores with the levels of anti-tTG IgA and anti-TGe IgA in DH and CS patients. Marsh scores for each DH (▲) and each CS patient (◆) were determined and plotted on the *x* axis. OD values for the corresponding anti-TGe IgA level (a) and for the corresponding anti-tTG IgA level (b) for each DH and each CS patient was plotted on the *y* axis. All patients were on a normal gluten-containing diet. Trend lines are provided for the DH patients (solid). For anti-TGe IgA in DH, $r=0.82$, for anti-tTG IgA in DH, $r=0.7$. For anti-TGe IgA in CS, $r=0.21$, for anti-tTG IgA in CS, $r=0.35$.

concentration of both antibodies in CS patients is independent of the degree of villous atrophy.

It is of great interest then, to understand how and why these antibodies are generated in both DH patients and celiac patients. Clearly, the consumption of gluten triggers symptoms in both diseases, but the mechanisms behind the production of these specific autoantibodies still remains enigmatic. It is possible that the catalyst(s) for the production of anti-tTG IgA and anti-TGe IgA in DH is (are) located in the intestine and is (are) associated with intestinal damage. Future work should be devoted to better understanding what these catalysts (beyond the ingestion of wheat) are. It would also be crucial to determine if the production of these transglutaminase-specific antibodies is necessary for the development of autoimmune pathology in both the small intestine and skin, or if they are they solely consequences of pathology in the intestine.

MATERIALS AND METHODS

Case definition

DH was diagnosed based upon the presence of pruritic papulovesicular lesions and the presence of granular IgA deposits in perilesional skin. Celiac sprue was defined as significant villous atrophy.

At least four endoscopic biopsies from distal duodenum were formalin fixed and paraffin embedded. Sections (5 μm) were stained with hematoxylin and eosin and scored based on the Marsh system.

Detection of anti-TGe IgA and anti-tTg IgA

Anti human tTG ELISA kits (The Binding Site Inc., San Diego, CA) and anti TGe ELISA kits (Alpco, Windham, NH) were used to measure the level of anti-tTG IgA and anti-TGe IgA in the sera of patients. All OD values were normalized by subtracting the value of the negative control provided in the kit. This would mean that any sample that had an OD value greater than the negative control was positive for the antibody tested.

Statistical analysis

Pearson's correlation coefficients were calculated using Microsoft Excel. This study was conducted according to the Declaration of Helsinki Principles and approved by the Mayo Foundation Institutional Review Board. Also, patients had provided consent before the study.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Elevation of IgA anti-epidermal transglutaminase antibodies in dermatitis herpetiformis

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Conflicts of interest
None declared.

Summary

Background Dermatitis herpetiformis (DH) is a papulovesicular eruption caused by ingestion of gluten. It is characterized by the deposition of IgA in the dermal papillae. IgA antibodies directed at tissue transglutaminase (TG2) are elevated in gluten-sensitive diseases including DH and coeliac disease (CD). More recently, antibodies directed at epidermal transglutaminase (TG3) were identified in patients with DH, and this may be the dominant autoantigen in this disease.
Objectives To measure IgA antibodies to TG3 and TG2 in patients with DH and CD, and control populations.
Methods Serum IgA antibodies against TG2 and TG3 were measured from adults with DH, adults and children with CD, patients with psoriasis, adult Red Cross blood donors, and paediatric controls.
Results Patients with DH and CD had elevated levels of IgA anti-TG2 antibodies compared with control populations. The levels in the patients with DH and adults with CD were similar. IgA anti-TG2 antibodies were higher in the children with CD compared with adults with DH and CD, and with control populations. Patients with DH and adults with CD had elevated levels of IgA anti-TG3 antibodies compared with children with CD and control populations. There was a trend towards higher levels in the patients with DH compared with adults with CD.
Conclusions IgA antibodies to TG3 are elevated in patients with DH and adults with CD. The progressive expansion of the epitope-binding profile of IgA antitransglutaminase antibodies in patients with CD may explain the development of DH in patients with undiagnosed CD during their adult life.

Dermatitis herpetiformis (DH) is a papulovesicular cutaneous eruption precipitated by ingestion of gluten.[1] Patients typically present with pruritic, excoriated papules on extensor surfaces of the elbows, knees, buttocks, back and scalp. This eruption is related to IgA deposits in the dermal papillae, a finding that is pathognomonic for DH.[2] These granular deposits of IgA are present in perilesional as well as uninvolved skin. The concentration of granular IgA at these sites decreases with adherence to a gluten-free diet, and reaccumulation occurs with the institution of a gluten-containing diet.[1] The antigenic specificity of the IgA in DH skin is unknown. In 2002 Sardy *et al.*[3] demonstrated the presence of epidermal transglutaminase (TG3) antigen within DH tissue. They proposed that this was the 'dermatitis herpetiformis autoantigen'. However, its relationship to disease activity and the mechanism by which it may participate in the production of the inflammatory process remain unclear. We recently produced a new anti-TG3 antibody and confirmed the observation of Sardy *et al.* that TG3 colocalizes with IgA in DH skin. TG3 was present in both inflamed, involved skin as well as uninvolved skin of patients with DH.[4] This indicates that the presence of the TG3 antigen is not the essential factor in disease pathogenesis. Therefore, TG3 is likely to play an important role in the pathogenesis of DH, but its exact role remains unknown.

Transglutaminases are a family of enzymes with pleiotropic functions, including protein crosslinking, deamidation, amine incorporation, esterification and hydrolysis.[5] There

are nine known human transglutaminases, and some are expressed in the epidermis. The functions of transglutaminases are important in multiple systems, including blood clotting, apoptosis and the maturation of the keratinocyte cytoskeleton. Genetic deficiency of TG2 causes lamellar ichthyosis, and other transglutaminases are also associated with genetic disease.[6]

DH is known to be associated with gluten-sensitive enteropathy (GSE) in virtually all cases.[7] However, the intestinal inflammatory process in DH is generally less severe than that seen in symptomatic coeliac disease (CD). Tissue transglutaminase (TG2), the antigen of an immune response in CD, is widely expressed in many tissues, including the gut epithelium.[5] The mechanism by which an immune response against TG2 is induced by gluten is unclear. However, gluten and gliadin proteins are glutamine rich and contain runs of polyglutamine. Glutens are also substrates of transglutaminase and it is hypothesized that a neoantigen is created by enzymatic modification of dietary gluten.[8] TG3 has a more restricted tissue expression. It also has low sequence homology with other transglutaminases and a relatively low conservation between species.[9] The trigger of the immune response to TG3 in the progression from CD to DH is unclear. However, it is likely that the disease-related antibodies are directed at post-translational epitopes.

The age at onset of CD is reported to be predominantly in early childhood with 70% of cases occurring before the age of 2 years,[10] although with increased sensitivity of serological assays more cases are now being diagnosed in adulthood. In contrast, the mean age at onset of DH is 38 years and childhood cases are very rare.[11]

We have hypothesized that IgA antibodies to TG3 are related to the inflammatory process, disease activity or development of inflammation over a period of time in patients with DH. To test this hypothesis we measured TG3 and TG2 IgA antibody levels by enzyme-linked immunosorbent assay (ELISA) in patients with DH, adults and children with CD and control populations including patients with psoriasis, Red Cross blood donors and paediatric controls.

Materials and methods

Study population

These studies were approved by the Institutional Review Board at the University of Utah. The patient groups in this study included 44 patients with newly diagnosed DH. These patients were a cross-section of our patients with clinical DH. To be considered eligible for the study, patients required a diagnosis of DH established by skin biopsy using direct immunofluorescence showing granular IgA deposits in dermal papillary tips. The age range for the patients with DH was 19–80 years. The first control group consisted of patients with psoriasis not known to have DH. This included 37 patients. The age range was not specified, but all were greater than 18 years. The second control group included 53 randomly ascertained Red Cross blood donors whose samples were collected without knowledge of any clinical symptoms. The age range was not specified, but all were greater than 18 years. The third control group included 50 randomly ascertained children from ARUP laboratories (Salt Lake City, UT, U.S.A.) aged 7–17 years without known DH or CD. Finally, we included 19 adults with new-onset CD and 16 children with new-onset CD. To be considered eligible for inclusion in the study, patients required a small bowel biopsy demonstrating evidence of CD. The duration and severity of clinical symptoms were unknown. The age range of the adults with CD was 20–79 years and that of the children with CD was 1–17 years.

All of the patients with DH and the adults and children with CD were on a normal, gluten-containing diet. No patients with DH were being treated with dapsone at the time of serum collection. None of the patients with DH, adults with CD or children with CD had partial or total IgA deficiency.

Tissue transglutaminase and epidermal transglutaminase assays

Serum IgA antibodies against TG2 and TG3 were measured using a commercially available ELISA assay. Serum was obtained from whole blood by centrifugation for 10 min and frozen at −80 °C with sodium azide. Semiquantitative detection of IgA anti-TG2 antibodies was performed per manufacturer protocol using the QUANTA Lite human recombinant TG2 IgA kits (INOVA Diagnostics, San Diego, CA, U.S.A.). Per manufacturer recommendations, positivity was assigned at ≥ 20 units. Semiquantitative detection of IgA antihuman recombinant TG3 was performed per manufacturer protocol using IgA anti-TG3 ELISA kits (Immundiagnostik, Bensheim, Germany). The upper limit of normal for this assay is assigned as 18 units. Assays were run in duplicate for all sera.

Statistical analysis

A univariate analysis of variance (ANOVA) was used to test the differences in mean IgA anti-TG2/TG3 values among the six groups (adult CD, DH, paediatric CD, psoriasis, Red Cross blood donor and paediatric control). Tukey's multiple comparison adjustment was further performed to determine which groups showed differences if an overall ANOVA was significant. In cases where data were skewed and did not have equal variance across groups, the response variables were then log-transformed and ANOVA was used on the transformed data. A nonparametric Kruskal–Wallis test was utilized to test the differences in IgA anti-TG2/TG3 among groups.

Results

Tissue transglutaminase

Patients with DH and CD had elevated levels of IgA anti-TG2 (Fig. 1). Twenty of 44 patients with DH (45%) had IgA

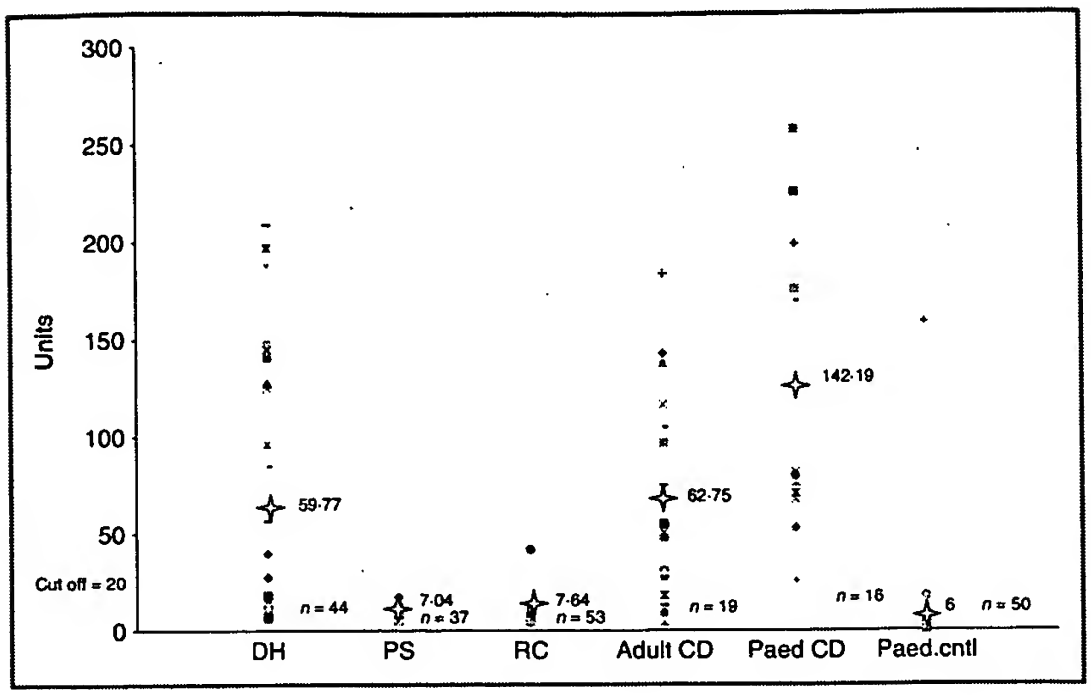

Fig 1. Tissue transglutaminase (TG2) values in 44 patients with dermatitis herpetiformis (DH), 37 patients with psoriasis (PS), 53 Red Cross blood donors (RC), 19 adults with coeliac disease (CD), 16 children with CD (Paed CD) and 50 paediatric controls (Paed.cntl). All patients were on normal, gluten-containing diets. Patients with DH and adults with CD had elevated levels of IgA anti-TG2 compared with Red Cross blood donors, patients with psoriasis and paediatric controls. Children with CD had higher levels of IgA anti-TG2 than both patients with DH and adults with CD. IgA anti-TG2 levels in adults with CD and patients with DH were similar. Mean values are indicated by a cross.

anti-TG2 levels > 20 units. The mean of our 44 patients was 59·77 (range 6–209). Likewise, 15 of 19 adults with CD (79%) had an elevation of IgA anti-TG2 with a mean of 62·75 (range 3–184). The children with CD had higher levels of IgA anti-TG2 (mean 142·19, range 26–258). All (n = 16) of these patients had elevated IgA anti-TG2 levels. In contrast, the group of patients with psoriasis had a mean level of 7·04 (range 5–7) comparable with the Red Cross blood donors (mean 7·64, range 5–42) and the paediatric control group (mean 6·0, range 0–159). There was one Red Cross blood donor and one paediatric control with an elevated IgA anti-TG2 level.

For statistical analysis, a univariate ANOVA was used to test the differences in mean IgA anti-TG2 values among groups. The differences in means in the six groups were highly significant ($P < 0·001$). Homogeneous subsets included (i) patients with psoriasis, Red Cross blood donors and paediatric controls; (ii) adult CD and adult DH; and (iii) paediatric CD, with no significant differences within a subset, and significant differences between subsets. Similar results were found when values were log-transformed and ANOVA used on the transformed data. Finally, a nonparametric Kruskal–Wallis test was performed to analyse the differences among values (not the means) among groups. There were significant differences among homogeneous groups (i, ii, iii) ($P < 0·001$).

Epidermal transglutaminase

Patients with DH had elevated levels of IgA anti-TG3 (Fig. 2). Twenty-three of 44 (52%) had IgA anti-TG3 levels > 18 units. The mean of our patients was 30·73 (range 7–153). Adults with CD also had elevated levels. Ten of 19 (53%) had elevated levels (mean 18·95, range 6·3–58). Children with CD did not have elevation of TG3. Only one of 16 (6%) had an elevated level (mean 9·02, range 3·92–21·14). The psoriatic, Red Cross blood donor and paediatric control populations had means of 6·23, 7·87 and 2·0, respectively. This was within the normal range. A single Red Cross blood donor had an elevated IgA anti-TG3 level of 20.

A univariate ANOVA was used to test the differences in mean IgA anti-TG3 values among groups. The differences among the groups were highly significant ($P < 0·001$). Homogeneous subsets were (i) psoriasis, Red Cross blood donors, paediatric controls and paediatric CD; and (ii) adult CD and adult DH, with no significant differences among subsets, and significant differences between subsets. When a Tukey's multiple comparison adjustment was made, the difference between adult CD and DH was close to statistical significance ($P = 0·053$). Similar results were seen on log-transformed data using ANOVA. A nonparametric Kruskal–Wallis test was used to analyse the differences in IgA anti-TG3 values (not means) among groups and showed significant differences among homogeneous groups (i, ii) ($P < 0·001$).

In summary, patients with DH and CD had comparable levels of IgA anti-TG2 antibodies. However, patients with DH had higher levels of IgA anti-TG3 antibodies, nearing traditional statistical significance ($P = 0·053$).

Discussion

We have evaluated the role of serum IgA antibody assays to TG2 and TG3 in patients with DH, adults and children with CD, and control populations. Perhaps most interesting is the fact that our children with CD had significantly lower levels of serum IgA anti-TG3 antibodies than our adults with CD. This occurred in the face of significantly elevated IgA anti-TG2 levels in the paediatric population compared with the adult population. There is an obvious dichotomy in the expression of the two antibody profiles in these age-controlled groups of patients with the same disorder. We find this particularly interesting because the average age at onset of DH in our patient population is 38 years.[11] The reason for the adult age at onset of DH compared with the frequent childhood onset of CD is unknown. The current data suggest to us that during childhood, patients with CD initially have elevated levels of

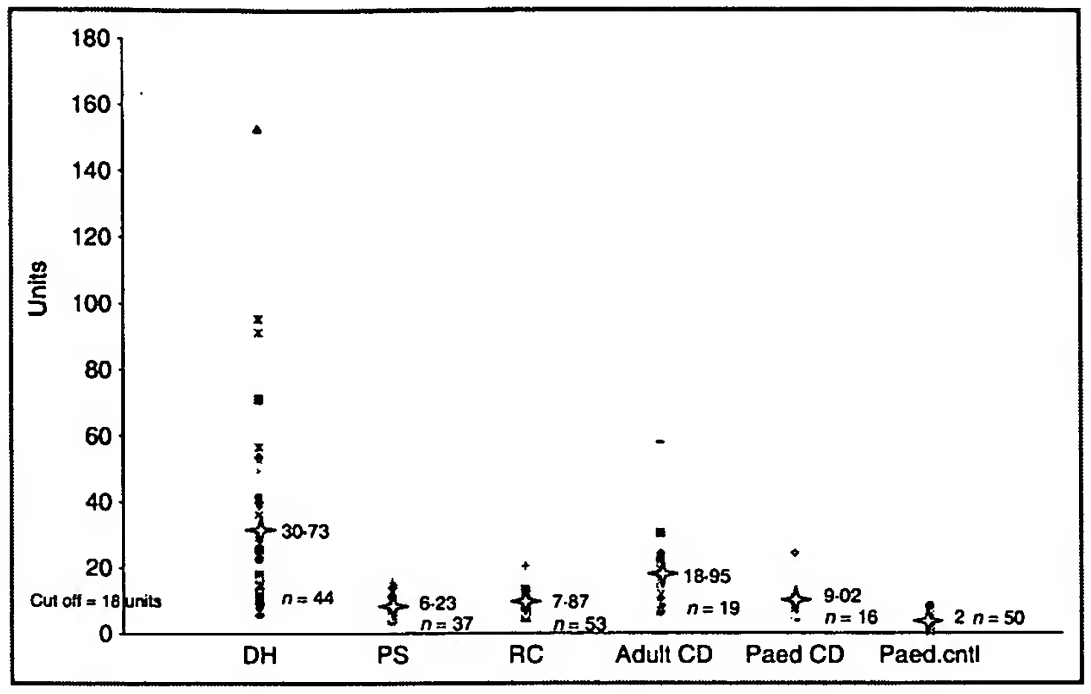

Fig 2. Epidermal transglutaminase (TG3) values in 44 patients with dermatitis herpetiformis (DH), 37 patients with psoriasis (PS), 53 Red Cross blood donors (RC), 19 adults with coeliac disease (CD), 16 children with CD (Paed CD) and 50 paediatric controls (Paed.cntl). All patients were on normal, gluten-containing diets. Patients with DH had elevated levels of IgA anti-TG3 compared with all other groups. Adults with CD had higher levels of IgA anti-TG3 than children with CD. There was a trend towards higher levels in patients with DH compared with adults with CD, but the results did not reach statistical significance (P = 0·053). Mean values are indicated by a cross.

IgA anti-TG2 antibodies but normal levels of IgA anti-TG3 antibodies. Although we do not have longitudinal studies of any individual patients, the data suggest to us that as time goes on and patients with untreated CD become adults, they develop elevated levels of IgA anti-TG3 antibodies compared with their childhood counterparts. We would propose that this group of patients with CD who develop elevated levels of IgA anti-TG3 antibodies over time comprises the patients at risk of developing DH in adulthood.

We have demonstrated that patients with DH have elevated levels of IgA anti-TG3 antibodies compared with control Red Cross blood donors. This has been described previously by Sardy *et al.*[3] However, Sardy *et al.* failed to note that the serum IgA anti-TG3 levels of patients with DH were higher than those of their control CD population. We tested 44 adults with DH for IgA anti-TG3 antibodies. Adults with DH and adults with CD both had elevated levels of IgA anti-TG3 antibodies compared with control groups and children with CD. There was a trend toward elevated levels of anti-TG3 antibodies in adults with DH compared with adults with CD although this did not quite reach statistical significance (P = 0·053). Perhaps a larger sample size would demonstrate significance.

In our study, approximately 50% of patients with DH were positive for IgA anti-TG3 antibodies. This suggests that factors other than IgA anti-TG3 antibodies are important in the disease pathogenesis of DH. It is known that IgA is present in DH skin for years and does not correlate with disease activity.[1] Circulating TG3 antibodies may fluctuate with gluten intake, intestinal inflammation or trauma to skin, and may be an indication of disease activity only at the time of serum collection. Further studies to correlate IgA anti-TG3 antibodies in patients with DH with disease parameters including disease duration, gluten intake, disease severity and dapsone dose are warranted to investigate this issue further.

In the TG2 studies we noted that both adults with DH and adults with CD had comparable levels of IgA anti-TG2 antibodies. This is similar to previously reported studies.[12,13] Interestingly, our paediatric CD population had higher levels of IgA anti-TG2 antibodies (P < 0·001) compared with adults with DH and CD. As expected, the serum IgA anti-TG2 levels of patients with psoriasis, Red Cross blood donors and paediatric controls were within the normal range. Rare patients with psoriasis have been said to have CD causally related to their psoriasis but this apparently did not influence our data.[14,15] These data provide further support for the evaluation of IgA anti-TG2 antibodies in patients with DH and CD and indicate that children with CD may have higher levels of these antibodies than their adult counterparts as well as adults with DH. These results warrant additional, larger studies in adults and children with CD specifically addressing any differences in IgA anti-TG2 or TG3 antibody levels, and any correlation of the antibody levels to severity of disease, duration of disease, or level of gluten restriction.

In summary, our data show the first evidence that progressive expansion of the epitope-binding profile of IgA antitransglutaminase antibodies may be the reason for development of DH in patients with undiagnosed GSE during their adult life.

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Epidermal Transglutaminase (TGase 3) Is the Autoantigen of Dermatitis Herpetiformis

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Abstract

Gluten sensitivity typically presents as celiac disease, a common chronic small intestinal disorder. However, in certain individuals it is associated with dermatitis herpetiformis, a blistering skin disease characterized by granular IgA deposits in the papillary dermis. While tissue transglutaminase has been implicated as the major autoantigen of gluten sensitive disease, there has been no explanation as to why this condition appears in two distinct forms. Here we show that while sera from patients with either form of gluten sensitive disease react both with tissue transglutaminase and the related enzyme epidermal (type 3) transglutaminase, antibodies in patients having dermatitis herpetiformis show a markedly higher avidity for epidermal transglutaminase. Further, these patients have an antibody population specific for this enzyme. We also show that the IgA precipitates in the papillary dermis of patients with dermatitis herpetiformis, the defining signs of the disease, contain epidermal transglutaminase, but not tissue transglutaminase or keratinocyte transglutaminase. These findings demonstrate that epidermal transglutaminase, rather than tissue transglutaminase, is the dominant autoantigen in dermatitis herpetiformis and explain why skin symptoms appear in a proportion of patients having gluten sensitive disease.

Key words: gluten sensitive enteropathy • celiac disease • IgA • immune complex • skin

Introduction

Gluten sensitive enteropathy (GSE)* is evoked and maintained by gluten, the adhesive mass of water-insoluble proteins found in many cereals. The clinical appearance of GSE is typically celiac disease (CD), a common chronic small bowel disorder; however, in certain individuals it is associated with the skin disorder dermatitis herpetiformis (DH). This is a bullous skin disease with polymorphic papules and blisters typically located over the extensor surfaces of the major joints and characterized by granular IgA deposits in the papillary dermis. Gastroenterological symptoms in DH are generally mild or clinically completely absent (1), however, inflammatory small bowel changes can often be found by histological examination even in the absence of clinical signs. The enteropathy in DH is morphologically identical with that in CD suggesting identical or very similar etiology and pathomechanism in both DH and CD (1). Further, both occur in the same genetic background being primarily associated with the HLA class II genes HLA-DQA1*0501, DQB1*02, and to a lesser extent with the HLA-DQA1*03, DQB1*0302 genes (for a review, see reference 2).

Both CD and DH patient sera show a typical IgA staining pattern when applied to tissue sections containing reticulin fibers such as endomysium. Recently, tissue transglutaminase (TGc, EC 2.3.2.13) was shown to be the predominant autoantigen in these sections (3, 4) and ELISA tests based upon this protein have been shown to be useful for the diagnosis of GSE (5, 6, 7, 8). TGc is a member of the transglutaminase (TG) family, which in man consists of nine distinct proteins present in a wide variety of cell types (Table I; references 9–27). TG family members show conservation especially of certain enzymatically relevant domains (10, 19). The active members catalyze a posttranslational modification linking low molecular weight amines to proteins, or induce an isopeptide bond between or within polypeptide chains leading to a cross-linked supramolecular

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*Abbreviations used in this paper: AU, arbitrary unit(s); CD, celiac disease; CI, confidence interval; DH, dermatitis herpetiformis; EMA, endomysium Ab; GSD, gluten sensitive disease; GSE, gluten sensitive enteropathy; TG, transglutaminase; TGc, tissue (cellular, type 2) transglutaminase; TGe, epidermal (type 3) transglutaminase; TGk, keratinocyte (type 1) transglutaminase; TGx, type 5 transglutaminase.

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Table I. *Comparison of Transglutaminases (References 9–27)*

TG	FXIIIa	TGk	TGc	TGe	TGp	TGx	TGy	TGz	Band 4.2
Gene name	F13A1	TGM1	TGM2	TGM3	TGM4	TGM5	TGM6	TGM7	EPB42
Chromosomal localization	6p24–25	14q11.2	20q11-12	20q11	3p21-22	15q15.2	20q11	15q15.2	15q15.2
Number of amino acids without the 1. Methionine	731	816	686	692	683	719	706	709	690
Molecular weight (kD)	~77	~106	~78	~77	~77	~81	~79	~80	~72
Primary molecular features	Exists as zymogen with 2 catalytic a subunits and 2 b subunits	Exists as zymogen, both cytosolic and membrane associated	Monomeric	Exists as zymogen	Monomeric	Monomeric (occurs as splice variants)	Unknown	Unknown	Monomeric, lacks enzymic activity
Presence in the skin (mRNA)	Yes	Yes	Yes	Yes	No	Yes	No?	Yes	Yes
Presence in other cells, tissues, or organs	Blood plasma, platelets, monocytes-macrophages, hepatocytes, chondrocytes, placenta	Keratinocytes, epithelial cells in stratified squamous epithelia, endometrium, ducts of pancreas and mammary glands	Widespread	Mouse: brain, stomach, spleen, small intestine, esophagus, testis, skeletal muscle, Human: kidney and lung.	Prostate only	Widespread	Unknown	Widespread	RBCs, platelets, fetal liver and kidney, adult brain, adult kidney?

FXIIIa, factor XIII a-subunit; TGy, transglutaminase type 6; TGz, transglutaminase type 7; band 4.2, erythrocyte protein band 4.2; RBC, red blood cell.

protein network (for reviews, see references 9 and 11); further, under special circumstances they are also able to deamidate glutamine residues.

The discovery of TGc as the main endomysial autoantigen failed to explain why only a proportion of gluten sensitive patients show symptoms of DH and whether there is a difference in the antigenic repertoire between CD and DH. By comparing the Ab responses to skin transglutaminases we could show that CD and DH are diseases where the main autoantigens are distinct but share common epitopes. This explains the similarities in pathology while also clarifying why certain gluten sensitive patients present with dermatological symptoms.

Materials and Methods

Mass spectrometry, SDS-PAGE, and endomysium Ab (EMA) tests were performed as described previously (8).

Production of Recombinant Human Transglutaminases. Human TGc was expressed recombinantly in the human embryonic kidney cell line 293-EBNA as a COOH-terminal fusion protein with the eight amino acid Strep II tag, and purified via streptavidin affinity chromatography as described previously (8).

To express human epidermal (type 3) transglutaminase (TGe), a method similar to that for TGc was used. Total RNA from human keratinocytes was reverse transcribed and the cDNA coding for the TGe proenzyme amplified by PCR using the forward primer 5′-ATTAAGCTTGCCGCCACCATGGCTGCTCTAGGAGTC, and the reverse primer 5′-ATTGCGGCCGCTTCGGCTACATCGATGGACAAC. The forward primer introduced a HindIII restriction site and a Kozak translation initiation sequence while the reverse primer inserted a NotI restriction site and removed the stop codon. The PCR product was digested with the HindIII/NotI restriction enzymes and inserted at the same restriction sites into the episomal eukaryotic expression vector pCEP-Pu/TGc/C-Strep (8), producing pCEP-Pu/TGe/C-Strep. The correct insertion and sequence of the full construct

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was verified by cycle sequencing. The plasmid was electroporated into human embryonic kidney cells (293-EBNA; Invitrogen) and transfected cells were selected with puromycin. Expression of the proenzyme, which has an additional COOH-terminal Strep II fusion tag, was confirmed by immunoblotting using a rabbit polyclonal serum raised against the Strep II tag (IBA). The protein was isolated by affinity chromatography using StrepTactin® (IBA) as described previously (8, 28).

Transglutaminase Activity Assay. TGe and TGc activity was measured by incorporation of [1,4-^{3}H]putrescine as described previously (8). The TGe was activated by partial proteolytic digestion preincubating it 20 min at 37°C together with either 45.4 μg/ml (0.5 U/ml) proteinase K (Sigma-Aldrich), or 45.4 μg/ml (55.4 U/ml) trypsin 1:250 (Sigma-Aldrich), or 1.18 mg/ml (1 U/ml) dispase (Life Technologies).

Production of Rabbit Sera against Human TGe. Rabbits were immunized with the COOH-terminally tagged human TGe proenzyme. TGe Abs were affinity purified by binding to Sepharose 6B (Amersham Pharmacia Biotech) coupled TGe and tested for cross-reactivity against TGc, keratinocyte TG (TGk), and factor XIII.

Sera and Patients. All patients had been examined at the Gastroenterological Departments of Internal Medicine or Pediatrics and the Department of Dermato-Venereology of Semmelweis University, Budapest. The diagnosis of CD was confirmed by EMA positivity and jejunal biopsy while DH was proven by skin biopsy using both conventional and immunohistochemical techniques. Sera were obtained from 59 patients with DH (including 43 samples from untreated patients, and 16 from patients on a complete or incomplete gluten-free diet) and 104 with CD (including 36 samples from untreated patients, and 68 from patients on a complete or incomplete gluten-free diet). Sera from 79 patients with non-CD gastrointestinal diseases, 47 with other diagnoses, and 30 from healthy individuals including 20 healthy relatives of CD patients were also included. Mean ages and sex ratios of the patients are detailed in Table II. No individual in this study had IgA deficiency. All serum samples were stored at −78°C until assayed.

TGe and TGc ELISAs. The ELISA method was as for the human TGc and described previously (8). Briefly: 96-well microtiter plates (MaxiSorp; Nunc) were coated with 1 μg per well of either human TGc or TGe in 100 μl of 50 mM Tris/HCl (pH 7.5) containing 5 mM $CaCl_2$ at 4°C overnight (at least 9 h). No blocking was used. After each step the wells were washed by 50 mM Tris/HCl (pH 7.5) containing 10 mM EDTA and 0.1% Tween 20 (TET). Sera were diluted to various concentrations with TET, and incubated on the plates for 1.5 h at room temperature. Bound IgA was detected by peroxidase-conjugated Ab against human IgA (Dako), diluted 1:4,000 in TET, and incubated for 1 h at room temperature. The color was developed by 100 μl of 60 μg/ml 3,3′,5,5′-tetramethylbenzidine substrate in 100 mM sodium acetate (pH 6.0) containing 0.015% H_2O_2 at room temperature. The reaction was stopped by adding 100 μl of 20% H_2SO_4. For the TGc, the color reaction was always stopped after 5 min; for the TGe, it was stopped after 15–20 min according to kinetic measurements so that the OD of the standard serum reached at least 0.6, but did not exceed 1.1. The absorbance was read in an ELISA reader at 450 nm. All serum samples were examined in triplicate, and triplicates of a negative and two positive reference sera were included in each assay. The Ab concentrations were expressed in arbitrary units (AU), i.e., as percentages of one of the positive reference sera. To semiquantitatively compare the IgA levels measured in the TGc and TGe ELISA assays, the standard serum was assayed in an identical manner against wells coated either with TGe or TGc. These were compared with standardized amounts of human IgA (Sigma-Aldrich). The protein coating efficiency of the ELISA plates was first determined by BCA protein quantification (Pierce Chemical Co.).

Inhibition ELISA. The ELISA method was as described above, but before their addition to the coated ELISA plate, the test sera were diluted to a fixed concentration and incubated with a dilution series of TGc or TGe. Sera and protein were mixed together for 90 min in a shaking incubator at 37°C. The fixed serum dilution was chosen in each case to obtain the greatest OD difference between the IgA Ab titers of the sera with and without

Table II. *The Patients' Number, Sex, Age at the Time of Blood Sampling, and Serum Ab Concentrations Against TGc and TGe*

Diagnosis	No. of patients	Male/ female	Mean age (yr) (min.–max.)	Median Ab cc. against TGc (in AU)		Median Ab cc. against TGe (in AU)	
				Median	95% CI	Median	95% CI
CD	104	40/64	12.5 (0.9–66)	—	—	—	—
CD, untreated	36	12/24	17.8 (1.4–66)	88.1	68.2–98.1	65.8	47.6–96.1
CD, on a GFD	68	28/40	9.7 (0.9–34.3)	22.7	16.6–41.0	20.3	15.2–26.6
DH	59	31/28	30.8 (6.2–73.5)	—	—	—	—
DH, untreated	43	23/20	32.8 (6.2–73.5)	63.3	54.8–77.6	70.1	54.6–75.7
DH, on a GFD	16	8/8	25.6 (10.3–45)	24.3	18.0–68.1	27.8	21.3–43.6
Controls	156	75/81	10.2 (0.5–55.5)	11.1	10.7–11.7	13.6	12.6–14.7
GI diseases	79	40/39	5.4 (0.5–27.6)	11.0	10.4–11.4	12.6	10.4–14.4
Other diagnoses	47	22/25	11.0 (0.7–53.2)	11.2	10.3–12.5	13.6	12.8–14.9
Healthy individuals	30	13/17	21.9 (0.7–55.5)	13.0	9.8–14.7	15.4	13.6–18.4
All samples	319	146/173	14.8 (0.5–73.5)	—	—	—	—

cc., concentrations; GFD, gluten-free diet; GI, gastrointestinal.

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preincubation (1:500–1:4,000 for inhibition of the TGc ELISA, 1:125–1:1,000 for the TGe ELISA). These diluted sera were then incubated with a dilution series containing different amounts of TGc or TGe in 160 μl TET. The color reaction was stopped at 5 min for the TGc-coated ELISA and 15 min for the TGe ELISA.

Affinity Purification of Patient Abs Directed Exclusively against TGe. TGc or TGe was coupled to CNBr activated Sepharose 4B, 50 μg of coupled protein per patient sample was used in the purifications described below. 80 μl of serum was diluted 1:10 with 10 mM Tris (pH 7.5) and was circulated over a TGc column for 1 h. For a number of high titer samples the efficiency of anti-TGc Ab depletion was assayed at this step. To obtain TGe specific Abs and remove any traces of TGc immunoreactivity, the flow through now depleted of TGc reacting Abs was then applied to a TGe column in the same manner. The TGe columns were washed with 250 μl of 10 mM Tris (pH 7.5) followed by 250 μl of 10 mM Tris, (pH 7.5) containing 0.5 M NaCl. Bound Abs were eluted with 250 μl 100 mM glycine (pH 2.5) or 250 μl 100 mM triethylamine (pH 11.5) directly into 250 μl 1 M Tris (pH 8.8). These solutions were then dialyzed against PBS (pH 7.4). The Abs eluted from the TGe column were tested in the TGc and TGe ELISAs as described above.

Direct Immunofluorescence. 6-μm cryostat tissue sections of human jejunal biopsy samples, human skin, or the aboral part of monkey esophagus were used for staining. Bound IgA was detected by α-chain specific, affinity purified, FITC-conjugated, goat anti–human IgA Abs (Sigma-Aldrich) at a dilution of 1:100 in phosphate-buffered saline (PBS, pH 7.4).

For localization of TGe, the affinity purified rabbit antisera raised against the recombinant TGe proenzyme was diluted 1:100 in PBS, followed by incubation with Cy3-labeled goat antisera raised against rabbit immunoglobulins (Sigma-Aldrich), diluted 1:800 in PBS. For TGc and TGk, mouse mAbs (Neomarkers, Ab-3 [a mix of mAbs CUB7402 and TG100], and Biomedical Technologies, mAb BC.1, respectively) were diluted 1:100 and 1:50 in PBS followed by incubation with Cy3- or FITC-labeled sheep anti–mouse Abs diluted 1:800 or 1:400, respectively.

Statistics. The optical densities (and thus titers given in AU values) had Gaussian distribution neither in the control group nor among CD or DH patients, thus for description of Ab concentrations, medians with their 95% confidence intervals (95% CI) are presented (29). For description and comparison of the two ELISA systems, the areas under the receiver operating characteristic curves are given. For comparison between patient groups, Mann-Whitney's nonparametric, unpaired, two-tailed test is shown (30). To describe the correlation of titers, the Spearman's correlation coefficient with its 95% CI and correlation analysis for unpaired data of nonnormal distribution was used (29, 30). For comparison of the TGc and TGe Ab inhibition assays, Wilcoxon's two-tailed signed rank test for pairs was performed (30).

Results

Production and Purification of Recombinant TGe

The human TGe was expressed in the 293-EBNA human embryonic kidney cell line as a fusion proenzyme with the Strep II tag. The protein was purified in a single step, and eluted as one 80 kD band when visualized by Coomassie-staining after SDS-PAGE (Fig. 1). The molecular mass calculated for the tagged human TGe proenzyme is 78.0 kD (the COOH-terminal tag having a mass of 1.2 kD). Although the recombinant human TGc and TGe have approximately the same calculated molecular mass (78.4 kD for TGc), the human TGc migrated slower than expected when visualized by SDS-PAGE (8; Fig. 1). Mass spectrometry of the TGe fusion protein gave a molecular mass of 77.8 kD. The yield from the lysate of a confluent cell monolayer in a cell culture dish of 13 cm diameter was ~100 μg of purified protein. In cell lysates, the activity of the recombinant human TGe was 2.5 times higher than the background activity of transglutaminases present in untransfected 293-EBNA cells. The freshly purified human TGe proenzyme showed ~2% of the activity of the same amount of human TGc. The human TGe activated with different proteases (proteinase K, trypsin, or dispase) showed similar or higher activity than the TGc, which is similar or higher than the activity of the commercially available guinea pig TGc enzyme (Sigma-Aldrich).

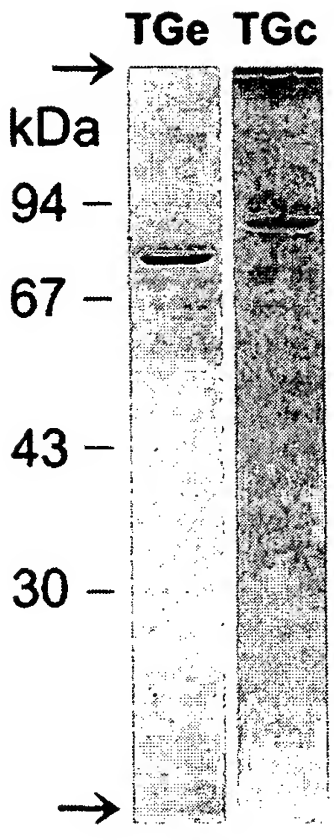

Figure 1. SDS-PAGE analysis of TGc and TGe after purification. Positions of molecular mass standards (kD) are indicated on the left. Arrows show the start and end lines of the gel.

CD and DH Patient Sera Contain IgA Abs against TGe and TGc

We have previously described a TGc ELISA based upon the human recombinant protein (8), ELISAs were performed against TGe or TGc using the same antigen concentration for coating, serum dilution, and positive and negative reference sera. As signals in the TGe ELISAs were for every serum significantly lower than against TGc, color development in the TGe ELISA was allowed to continue 3–4 times longer than that in the TGc ELISA until the positive reference serum reached similar ODs in both ELISAs. The results are expressed as a percentage (AU) of the signal of the positive reference serum in both assays. These ELISA results show that patients with both DH and CD have serum IgA Abs reacting against TGe and TGc (Fig. 2). To allow a comparison of the levels of IgA directed against either TGc or TGe, the reference serum (100 AU in the above assays) was assayed under identical conditions upon

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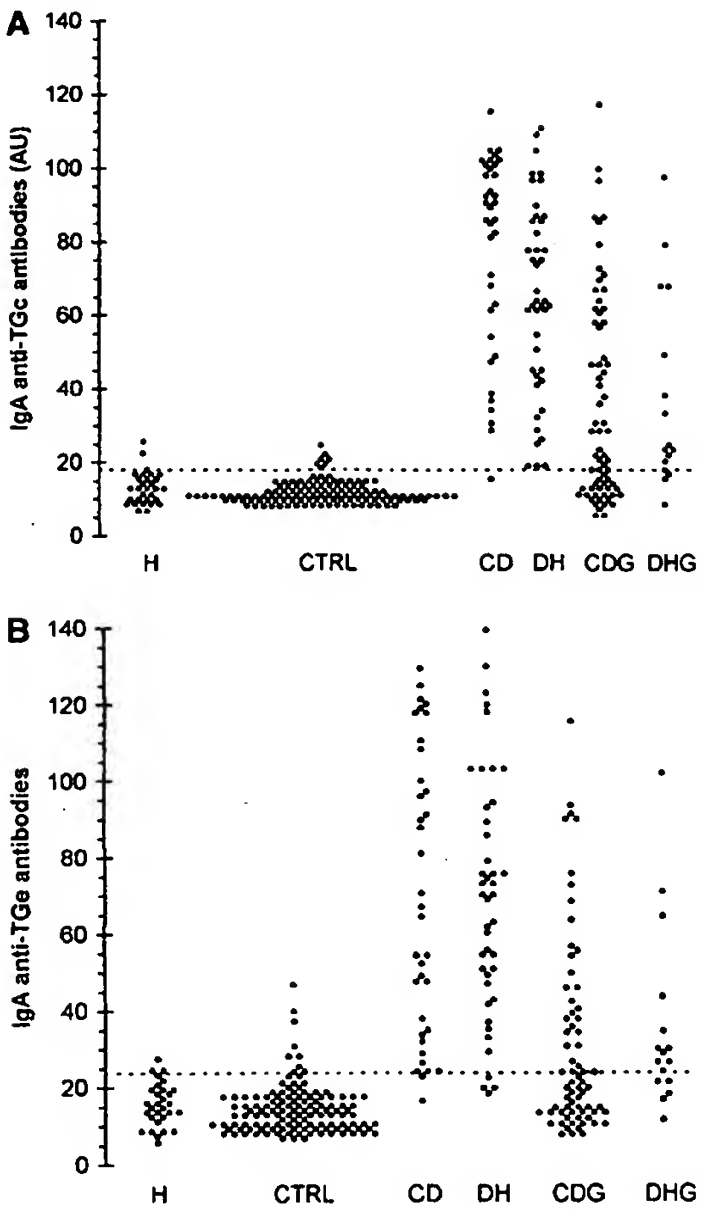

Figure 2. Analysis of serum anti-TGe and anti-TGc IgA. Serum concentrations of IgA Abs (in AU) against human TGc (A) and TGe (B) in healthy individuals (H), other controls (CTRL), patients having untreated CD or DH, as well as those on a complete or incomplete gluten-free diet (CDG and DHG, respectively). 100 AU corresponds to 16.78 μg IgA/ml of serum in the TGc ELISA and 2.45 μg IgA/ml in the TGe ELISA.

TGe and TGc coated ELISA wells in parallel to wells coated with a known amount of IgA. The anti-TGc IgA signal corresponded to 16.78 μg/ml of serum while the concentration of anti-TGe IgA in the positive reference serum was 2.45 μg/ml. In the TGe ELISA, the mean intra- and interassay coefficients of variation for the positive standard serum used for AU calculation were 2.7 and 19.1%, respectively. The mean intra- and interassay coefficients of variation (using Ab concentrations given in AU) for the other sera tested in the human TGe ELISA were 4.7% (n = 334) and 16.4% (n = 74), respectively.

The median Ab concentrations (in AUs) from the TGe and TGc ELISAs with their 95% CIs are presented in Table II. Although the confidence intervals overlapped, the median Ab concentration against TGc was significantly higher in CD than in DH patients (P = 0.0188). However, there was no significant difference in the Ab levels against TGe between CD and DH patients. The median Ab concentrations against TGc and TGe were significantly higher in untreated CD or DH patients when compared with the controls ($P < 0.0001$ in each case). Differences between the control subgroups were not significant. Both CD and DH patients had reduced Ab activity against TGe when on a gluten-free diet, results similar to those observed for TGc Abs.

The two ELISAs showed good linear correlation (r_S = 0.851, 95% CI: 0.818–0.878, $P < 0.0001$, data not shown). Indeed, the human TGe ELISA seemed to be suitable for diagnosis of GSE. The area under the receiver operating characteristic curve was 0.982 (in the TGc ELISA it was 0.997). In the TGe ELISA, a cutoff value of 23.7 AU, chosen based upon the analysis of the receiver operating characteristic curve, gave a specificity and a sensitivity of 92.3% (95% CI: 88.9–95.7%) and 92.4% (95% CI: 89–95.8%), respectively. The coincidence of the human TGe assay with the clinical diagnosis of CD or DH was 217/235 (92.3%), giving 12 false-positive and 6 false-negative results (Fig. 2 B). Four of the false-negative patients had DH, two of them were EMA negative. All the other DH or CD patients were positive for EMA.

For comparison, the TGc ELISA using a cut-off value of 18 AU (8) gave in this study a specificity and a sensitivity of 94.2% (95% CI: 91.2–97.2%) and 98.7% (95% CI: 97.2–100%), respectively. The coincidence of the human TGc assay with the clinical diagnosis was 225/235 (95.7%), giving one false-negative and nine false-positive results (Fig. 2 A). The false-negative serum and three of the false-positive sera were also falsely detected in the TGe ELISA.

These results suggest that either GSE patients have Abs cross-reacting between different transglutaminases or that specific Abs against both TGc and TGe occur in GSE and that Abs directed against TGe, as those against TGc, are maintained by the ingestion of gluten.

Inhibition ELISAs Show Differences in Ab Avidity to TGe between DH and CD Patients

To discover the significance of Ab cross-reactivity between these enzymes within the two patient groups, we performed inhibition studies. ELISA plates were coated with either human TGc or TGe, and the patient sera were preincubated with various concentrations of either of the two transglutaminases. Initial experiments allowed us to find appropriate serum dilutions giving results within a linear range for the given ELISA. The degree of inhibition produced by the preincubation with either of the two proteins was compared with control samples where the sera had been preincubated with buffer alone. The results are presented as reduction in the optical density given as percentage of the controls. Two examples of these inhibition ELISAs performed over a range of inhibitor concentrations with typical CD and DH sera are shown in Fig. 3. For group analysis of 36 CD and 34 DH patients, results of inhibition with 32 ng and 1 μg of the relevant transglutaminase are shown in Fig. 4.

Inhibition of Abs against TGc. We analyzed 34 sera of DH patients and 36 CD patients. The sera were diluted as described above and preincubated with human TGc or TGe

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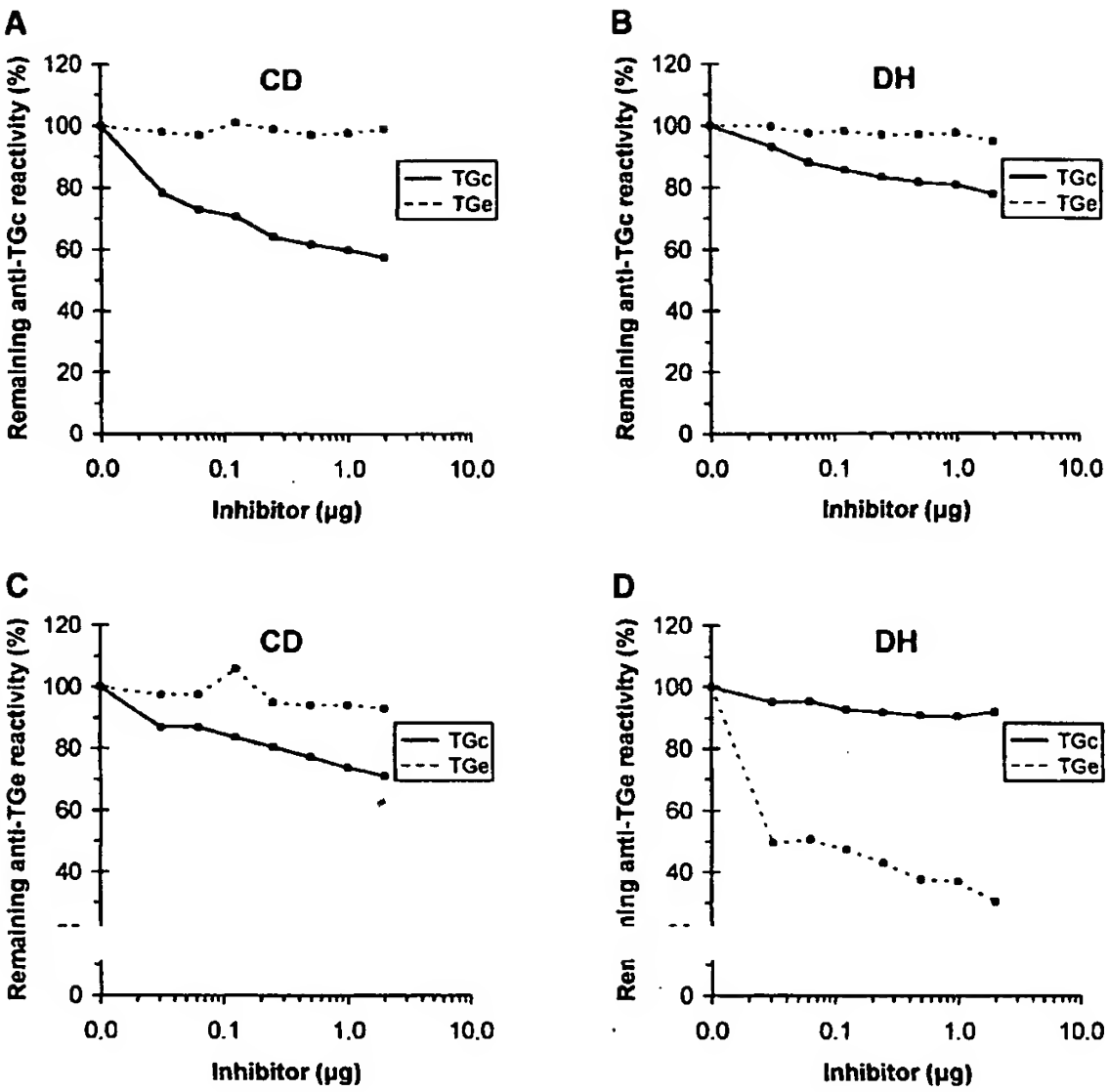

Figure 3. Transglutaminase inhibition ELISAs, typical examples of inhibition curves. Each diagram shows the effect of preincubation on the remaining IgA Ab reactivity in one single serum sample from a patient with untreated CD (A and C) or DH (B and D). On the vertical axis is the remaining IgA Ab reactivity against TGc (A and B) or TGe (C and D) given in percentage of the buffer control, on the horizontal axis are inhibitor amounts on a logarithmic scale used for preincubation. The control was preincubated with buffer only, the other samples with a serial dilution of TGc (continuous line) or TGe (dashed line). The TGe is seen to be an effective inhibitor of IgA Abs against TGe only in DH patients (D), but not in individuals with CD (C) (see group analysis in Fig. 4, statistics in the text). TGc has the greatest inhibitory effect on IgA Abs against TGc in CD patients (A).

in various concentrations before addition to ELISA wells coated with TGc. While incubation with even such low amounts as 32 ng of TGc (always in a volume of 160 µl, see Materials and Methods for details) effectively inhibited the reactivity of the sera from both the DH and CD patients with the coated TGc, the TGe failed to block the TGc reactivity (Fig. 3, A and B). Only at very high concentrations (above 10 µg) did preincubation with TGe produce any inhibition in the TGc ELISA (data not shown). There was no significant difference in the inhibition when comparing the results from DH or CD patient groups in these experiments.

Inhibition of Abs against TGe. Sera from DH and CD patients were diluted to the chosen dilution and preincubated with TGc or TGe before addition to ELISA wells coated with TGe. Here the human TGe, at 32 ng, effectively inhibited the reactivity of sera from DH patients with human TGe (Figs. 3 D and 4), but failed to inhibit that from CD patients (Figs. 3 C and 4). At higher concentrations, the inhibitory effect of preincubation with TGe increased with sera from DH patients and also a slight inhibition of the reactivity of sera from CD patients occurred which was more apparent upon the addition of very high amounts of TGe (up to 8 µg, results not shown). The difference between CD and DH patient groups upon inhibition with TGe was highly significant ($P < 0.0001$; Fig. 4).

At low concentrations, the human TGc produced only very marginal inhibition. However, when at high concentrations (at or above 1 µg), it could inhibit the reactivity of IgA Abs to TGe in both disease groups (Fig. 3, C–D, and

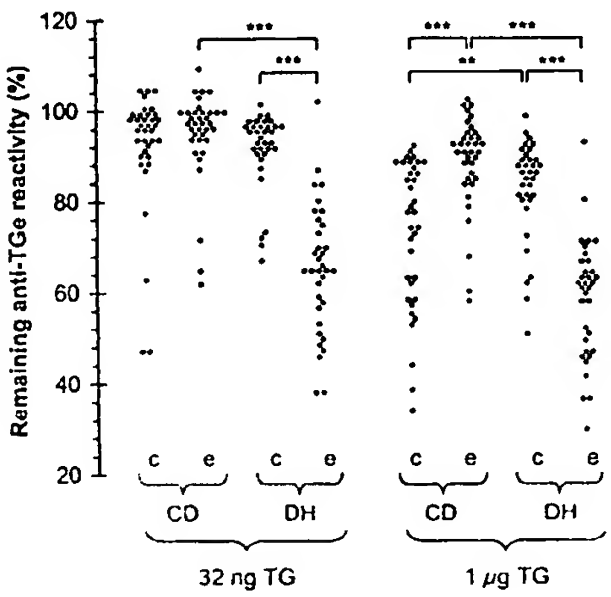

Figure 4. Effect of preincubation of sera from patients with CD ($n = 36$) or DH ($n = 34$). On the vertical axis, remaining IgA Ab reactivity against TGe is indicated in percentage of the buffer control. The four dot diagrams on the left show the inhibitory effect of preincubation with 32 ng of TGc (c) or TGe (e) on the remaining IgA Ab reactivity of sera from patients with CD and DH. The four dot diagrams on the right demonstrate the same using 1 µg of TGc or TGe for preincubation. The asterisks on top of connecting lines show the degree of significance in the difference between the two groups of samples so linked: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

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Fig. 4), although CD sera were more strongly inhibited than DH sera ($P \leq 0.0054$). These results provide evidence of various IgA Ab populations directed against both common and different epitopes on the two molecules and suggest that in DH patients there are IgA Abs with a high avidity directed against TGe.

Purification of TGe Abs from DH Sera

To discover if there are Ab populations exclusively directed against TGe present in either DH or CD patients, we affinity purified TGe specific Abs from patient sera. Sera from 20 CD patients and 18 DH patients were applied to columns of Sepharose 4B to which TGc had been covalently coupled. To test the efficiency of the removal of TGc Abs from the sera, the flow through fractions from this column were compared with a dilution series of the starting sera. This was performed for a number of high titer sera and showed a reduction of the TGc titer by some 98–99%. To isolate anti-TGe IgA, the immunodepleted (flow through) fraction was applied to columns carrying TGe, and after washing, the Abs binding to TGe were eluted. The eluates were compared with the unprocessed, precolumn sera for anti-TGe and anti-TGc immunoreactivity in the relevant ELISAs.

The removal of TGc immunoreactivity was highly effective with the eluates from the TGe column showing little or no reactivity in the TGc ELISA (Fig. 5). In the TGe ELISA, however, the eluates from DH patients showed in almost all cases significant levels of TGe immunoreactivity, while those of CD patients generally failed to give a signal (Fig. 5) displaying clear evidence for the existence of a TGe-specific Ab population which is usually present in DH but absent from the vast majority of CD patients.

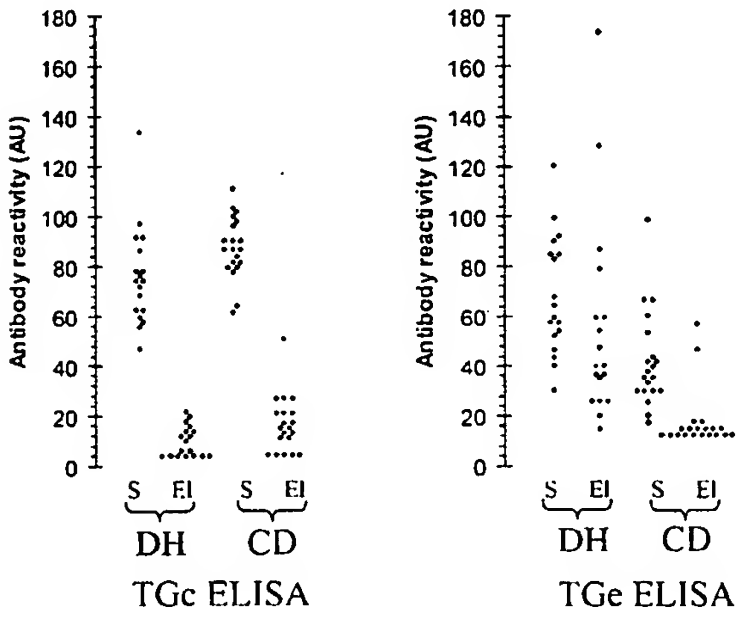

Figure 5. Affinity purification of the Ab population directed against TGe. Sera from CD and DH patients were circulated over columns of Sepharose 4B to which TGc had been covalently coupled. The immunodepleted fraction was then applied to columns carrying TGe. Unprocessed serum samples (S) and eluates from the washed TGe column (El) were compared for TGe and TGc immunoreactivity. The depletion worked with high efficiency and there was little or no reactivity in the TGc ELISA. In the TGe ELISA, the eluates from DH patients showed significant levels of TGe immunoreactivity, while those of CD patients generally failed to give a signal. Hence patients with DH have significant levels of Abs directed specifically against TGe which do not cross react with TGc, these Abs are absent in CD.

TGe Is Present within the IgA Precipitates in DH Skin

The antigenic component of the granular IgA precipitates occurring in the skin of DH patients was investigated for the presence of transglutaminases. A rabbit antiserum directed against the tagged human recombinant TGe proenzyme was produced and the specificity of the purified antiserum was verified in ELISA and immunoblots. This serum gave no cross-reaction with human TGk or factor XIIIa and a slight reactivity to human TGc, which was approximately a 100-fold lower than that to TGe. In normal human skin, the anti-TGe Ab stained solely the epidermis in a tapering manner being most intensive in the upper keratinocyte layers and quite different to the expression seen for TGc (results not shown). In addition to the epidermal signals, identical to those seen in normal skin, immunostaining of the skin from 8 DH patients with this antiserum revealed that TGe is found in aggregates within the dermal papillae (Fig. 6, A and C). This staining could be blocked efficiently by preincubation of the sera with TGe but not TGc or TGk (results not shown). Dual staining for the presence of IgA showed that TGe and IgA colocalized within these precipitates (Fig. 6 A) while TGc and TGk were absent from the aggregates and had the expression pattern seen in normal skin (TGc: Fig. 6 C; TGk: not shown). To verify that an alteration in the TGe staining pattern is a specific finding for DH skin, skin from patients suffering from linear IgA dermatosis was also analyzed. In this disease, IgA also accumulates within the skin but binds to the dermo-epidermal basement membrane. Here no TGe staining of the dermis or at the basement membrane was found and no colocalization with the IgA signal occurred (Fig. 6 B). Hence the IgA precipitates found in DH are immunocomplexes containing TGe which accumulate specifically in this disease.

Discussion

CD and DH are closely related diseases both induced by a sensitivity to gluten. As they share an identical jejunal pathology, genetic background, similar pathomechanism, common diagnostic analysis, and shared dietary possibilities for therapy, we suggest the term "gluten sensitive disease" (GSD) for both of these forms of condition showing manifestation on gluten challenge, disappearance of symptoms on gluten withdrawal, and recurrence of disease upon gluten intake. Both forms of GSD can be subdivided into clinical manifestations of different severity, as they can also present with unspecific or even absent clinical signs or symptoms. Moreover, epidemiological studies show that the majority of GSD patients actually have very mild or atypical symptoms or often clinically silent disease (31).

GSD is the result of three processes culminating in the intestinal mucosal damage of CD and in the skin defects of DH. Both are hereditable conditions with strong associa-

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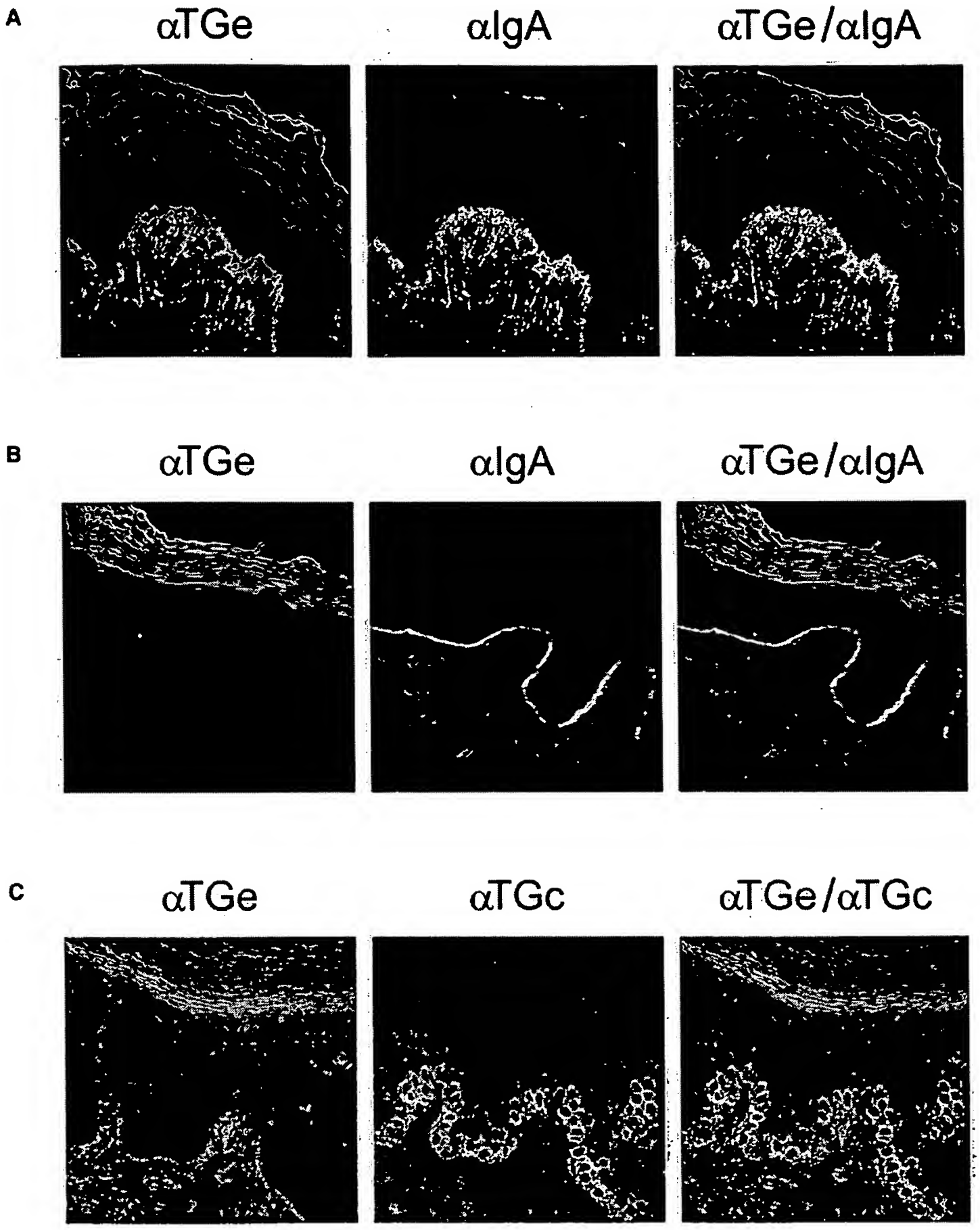

Figure 6. Colocalization of IgA and TGe in the papillary dermis of DH patients. (A) In DH patients, TGe shows normal distribution pattern in the epidermis; typical precipitates are present, however, in the upper dermis. In these precipitates, the TGe (red) and the IgA (green) colocalize as visualized with a confocal microscope (yellow). (B) The TGe precipitates are absent in linear IgA dermatosis, a bullous skin disease with linear IgA precipitates in the upper dermis, suggesting that TGe deposits are specific markers for DH. In addition, this also demonstrates the absence of cross-reactivity of anti-TGe Abs with IgA. (C) The TGe (and the IgA, data not shown) precipitates do not colocalize with TGc.

tions to identical HLA haplotypes; however, it does not appear that genetic factors alone decide the clinical outcome as monozygotic twins may exhibit any combination of manifest CD, DH, or clinically silent GSD (1, 32) proving environmental factors are also significant. The main environmental factor in GSD is the ingestion of gluten in cereals, however, Abs against these proteins are not perfect diagnostic markers for the disease and are found in a range of other

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gastrointestinal disorders without any evidence that they play a pathogenic role in these conditions (33, 34). However, the third factor, namely that the patients' immune system produces Abs reacting with the endomysium, is found present in every form of GSD and is highly specific (35). The standard serological diagnosis for GSD depends upon the staining of endomysial tissue with IgA Abs, and it was shown that the antigen within these sections is TGc (3, 6, 7, 36, 37). The presence of autoantibodies to TGc has been shown to be linked to disease activity with the titer decreasing when patients are placed upon a gluten free diet and increasing upon subsequent gluten challenge (6, 7). Our aim was to understand why GSD appears as two distinct clinical entities.

Our initial hypothesis was that there was immunoreactivity specifically in the DH patient population against a further transglutaminase expressed in the skin. Four transglutaminases have been isolated from the skin, TGe and TGk are both produced by epidermal cells, as is TGc, which is also found together with factor XIIIa in the dermis. To discover if any of these proteins are antigens in DH we produced ELISAs based upon human transglutaminases. Initial ELISA studies using human recombinant TGk as well as the commercially available human factor XIIIa showed that there was no specific immunoreactivity in either CD or DH patient sera against these enzymes (results not shown). However, both patient groups had Abs recognizing TGe as well as TGc. The results from the TGc and TGe ELISAs showed a good correlation and indeed the specificity and sensitivity of the TGe ELISA came close to that of the TGc based test. However, in CD patients, the median Ab concentration against TGc was higher than against TGe, and this was reversed for DH patients (Table II), although because of the overlapping confidence intervals this tendency cannot be judged to be a true distinction. Further, the immunoreactivity for both proteins and in both disease groups showed a reduction in titer when the patients were placed upon a gluten free diet. This is in agreement with known clinical improvement seen in DH patients on a gluten free diet and the common background of both diseases.

Members of the transglutaminase family share a high degree of sequence conservation especially in their active sites. In the case of the TGe and TGc there is an overall conservation of 38% at the amino acid level, but with up to 64% homology in certain regions (19). Phylogenetically, TGe and TGc seem to be more related to each other than to TGk or factor XIIIa (10). Cross-reacting Abs against TGc or TGe in GSD patients are therefore not surprising; however, we could use ELISA blocking experiments to show differences in avidity for the different TGs between the two patients groups. As expected, TGc inhibited the reactivity of the sera from both CD and DH patients in the TGc ELISAs showing that anti-TGc immunoglobulin species are present in both diseases. In the TGe ELISA, however, inhibition with TGe could be invoked only in DH patient sera suggesting the presence of high affinity anti-TGe Abs in DH, and the presence of only of low affinity TGe reactive Abs in CD. Recently three new members of the TG gene family, type 5 transglutaminase (TGx), TGy, and TGz (Table I), have been described (10). We were unable to test these transglutaminases in our study, thus the possibility of cross-reactivity with other TGs cannot be completely excluded.

Our results prove the presence of two Ab populations in GSD, one against only TGe (detected in patients with DH only, see Fig. 5), and one directed against common epitopes of TGe and TGc (detected in both CD and DH, see Fig. 4). A third population against only TGc may also be present, but was not investigated. As shown by the differences in the IgA levels against TGc and TGe in the standard serum, the concentration of IgA Abs directed against epitopes present on TGe is much lower than that directed against TGc. This means that both in DH and CD patients, only a fraction of the Abs directed primarily against TGc have cross-reactivity with TGe. In addition, DH patients develop a higher avidity Ab population directed against only TGe. This Ab fraction also is much smaller than that against TGc. This explains why there is no apparent difference between sera of CD or DH patient groups in either the TGc or TGe ELISAs (Fig. 2, A and B) and why the TGc and TGe ELISA results from patient sera correlate. While the Ab population directed against only TGe (found in DH patients and having high avidity), can be inhibited with very small amounts of TGe, those primarily directed against TGc, (having low avidity against TGe) can only be inhibited with high amounts of TGe. Accordingly in DH patients typically a two-step inhibition curve is seen (Fig. 3 D). This further explains why preincubation of CD serum (which has little or no high avidity TGe Abs) with TGc has a greater impact on reactivity to TGe than preincubation with TGe itself (Fig. 3 C).

While affinity purification of sera of GSD patients showed that the presence of TGe-specific IgA is a hallmark of DH rather than CD, a small number of patients (10%) deviated from the bulk of results in both the blocking assay and in their behavior upon purification. These DH patients, having Ab response characteristic for CD, might currently be showing transition from CD into DH. The CD patients, behaving rather as expected for DH patients, might be expected in later life to show symptoms of DH, if they continue gluten intake.

The diagnosis of DH depends upon the finding of IgA deposits within the dermal papillae, and in the majority of patients EMAs can also be detected (38). The latter is typically shown with the labeling of the endomysium, which has earlier been shown to colocalize with the TGc staining pattern (6, 37). Our observation that the major Ab population both in CD and in DH is directed against TGc, supports the finding that the endomysial signal seen on monkey esophagus is of TGc origin and indeed we found that TGe was present only in the epithelial cells of monkey esophagus, but not in the endomysium (results not shown). The epidermal staining pattern with the TGe antiserum supported previous reports on its distribution (39), TGe being present in the epidermis in a tapering manner, with maximum staining of the upper epidermal layers. It was also found present in hair follicles. In DH skin, the epider-

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mal and hair follicle staining was indistinguishable from that seen in normal skin, but the dermal IgA containing aggregates also stained strongly for TGe. TGc, which was found present in the basal keratinocytes, was absent from these aggregates.

Our hypothesis for the etiology and pathogenesis of DH is that TGc-gluten complexes initiate an IgA autoantibody response (40), but fail to produce high affinity anti-TGc immunoglobulins, so resulting initially in a silent CD. These Abs cross react with TGe, but are of low avidity to it. After prolonged gliadin provocation (DH patients usually show symptoms later in life than CD patients), specific cross-reacting Ab populations develop in patients who will go on to acquire DH. These Abs have a low affinity to TGc, but extremely high affinity to TGe. Whether they arise against TGe as a primary antigen or are the result of epitope spreading cannot be answered at the moment. Why only a proportion of patients develop specific Abs against TGe and why these patients show only a very mild form of enteropathy also remains to be elucidated.

We speculate that the skin pathology may be evoked by the dermal deposition of circulating immune complexes containing IgA and TGe. Possibly the TGe is active, resulting in covalent cross-linking of the complex to certain dermal structural elements. This could be the basis for the stability of these immune complexes, as it is known that the IgA deposits in DH skin stay detectable up to a decade after the introduction of a completely gluten-free diet (1). It would also explain why it has not been possible to extract the IgA immune complexes from the skin of DH patients. Inflammation of the skin might eliminate the covalently bound immune complexes. Indeed often the IgA granules are present perilesionally but not in areas of blister formation. This circulating immune complex hypothesis for DH is supported by a number of findings. First, TGe is expressed in several tissues in the body (Table I), and thus the antigen might originate from organs other than the skin. We failed to detect TGe in the human jejunum with our rabbit antiserum (results not shown), although the mRNA for the TGe proenzyme was demonstrated in mouse jejunal tissue extracts (17). We did, however, detect TGe mRNA in other human organs including the kidney (results not shown). Further, the skin histology in DH has features in common with other dermatoses induced by circulating immune complexes (41), and although the main site of immune complex deposition is the upper dermis, they are also present in vessel walls. In DH, asymptomatic IgA immune complex depositions can be detected in the kidney (42), a situation often seen in systemic diseases caused by circulating immune complexes, and indeed DH-associated IgA nephropathy has been reported (43). The fact that Abs in DH sera do not bind to the normal human papillary dermis again suggests that the deposits derive from circulating immune complexes. The factors that induce the classical distribution pattern of skin lesions in DH patients, localized mainly on extensor aspects, are as yet unknown. Here, however, we have shown that high affinity anti-TGe IgA maintained by gluten is present in DH patients and not in patients suffering from CD and that TGe is present in the skin IgA aggregates typical of DH.

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